IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: LAPIDOT=2

In re Application of:	)	Art Unit: 1632
LAPIDOT et al	)	Examiner: A. M. S. Wehbe
Appln. No.: 09/744,654	)	Washington, D.C.
Date Filed: July 29, 2001	)	Confirmation No.: 2645
For: HEMATOPOIETIC CELL COMPOSITION FOR USE IN...	)	May 16, 2005

DECLARATION UNDER 37 CFR §1.132

U.S. Patent and Trademark Office
2011 South Clark Place
Customer Window, **Mail Stop**
Crystal Plaza Two, Lobby, Room 1B03
Arlington, Virginia 22202

Sir:

I, Tsvee LAPIDOT, hereby declare and state as follows:

I am a coinventor of the above-identified application and my educational and professional experience is presented in the curriculum vitae attached hereto.

It is my understanding that the claims in the above-identified application have been rejected under 35 U.S.C. §103(a) as being unpatentable/obvious over Kanz et al., U.S. Patent No. 5,541,103, in view of Mohle et al., *Blood* 91(12):4523-4530 (1998).

The experiments described in Examples 3 and 4 of the present specification and described below, which demonstrate unexpected results, were conducted by me or under my direct supervision, and I can attest of my own personal knowledge that all the results reported herein and in Examples 3 and 4 of the present specification are true and accurate.

The materials and methods section for the Examples in the present specification on pages 24-29 provide the experimental methods used to obtain the results reported in Examples 3 and 4 and further discussed below. The figures discussed below refer to the figures from the instant application.

For the sake of clarity, before describing in detail our results disclosed in application No. 09/744,654, I would like to discuss our animal model of transplantation (NOD/SCID mice) and the human cells that we used in this model of transplantation.

NOD/SCID (non-obese diabetes/severe combined immunodeficient) mice are an outbreed strain that have multiple immunological defects and therefore permit xenogeneic transplantation (i.e. transplantation from an organism of different species). As such, NOD/SCID mice were used in our experiments to check homing and repopulation of purified human hematopoietic subsets of cells, which include immature human

CD34+ cells. Immature human CD34+ cells consist of a heterogeneous population which contains a majority of more differentiated, CD38+ cells containing high levels of CD38 (about 80% of all cord blood (CB) CD34+ cells), a minority of primitive, less differentiated $CD38^{low}$ cells with only low levels of CD38 expression (up to 15% of all CB CD34+ cells), and a smaller minority of more primitive undifferentiated $CD34+/CD38^-$ cells without detectable levels of CD38 expression (up to 5% of all CB CD34+ cells). Single cells with $CD34+/CD38^{-/low}$ phenotype can differentiate to all lineages, suggesting that they also contain pluripotent stem cells.

The present patent application relates to a method that allows fast preparation of a cell composition comprising primitive stem cells capable of enhanced self-renewal in vivo and which exhibit high migratory and engraftment capability. This method comprises the steps of a short-term (up to 5 days) stimulation of an initial cell composition, comprising human stem cells, with a suitable agent capable of converting primitive cell $CD38^{-/low}$ $CXCR4^{-/low}$ into a $CD38^{-/low}$ $CXCR4^{+}$ stem cells, and the step of selection of only those cells that migrate in response to SDF-1. The first step of our method increases surface CXCR4 expression in the initial composition of cells, and the second step selects for cells having functionally active CXCR4.

Among the agents capable of increasing surface CXCR4 are lectins, cytokines, stroma cells or a mixture thereof. In some examples, we used stem cell factor (SCF) as the stimulatory agent that induces CXCR4 expression, and immature human CD34+ cells (from adult mobilized peripheral blood [PBL], which have less $CD38^-$ cells and much less repopulation potential compared to cord blood cells) as the initial cell composition. Following a short stimulation of human CD34+ cells with SCF (40 hours), we showed that the amount of the initial CD34+ cells (Fig. 3A, the area under the graphs) before (Fig. 3A graph b) and after a short-term SCF-treatment remained almost the same (Fig. 3A graph c). Therefore, we demonstrated that the short SCF stimulation used in the first step of our method did not induce cell expansion. However, we demonstrated that the same short-term SCF-stimulation, that did not cause cell expansion, induced a dramatic increase in: CXCR4 expression (see Fig. 3A, graph c, the graph c with SCF-treated cells is shifted to the right), percentage of CXCR4+ cells, migration and engraftment (Figs. 3A-C). In a further experiment we compared engraftment of a given amount of non-stimulated human CD34+ cells versus engraftment of half of the amount of human CD34+ cells stimulated with SCF (Fig. 3D, a better quality copy of which is found as Fig. 3D in the Peled et al, Science 283:845-848, 1999). The results obtained in

said experiment show superior engraftment with only half the amount of SCF treated cells compared to non-treated cells.

The increase in CXCR4 mediated by SCF stimulation was shown to be necessary for better engraftment, since we showed that the positive effect of SCF stimulation on engraftment could be prevented if following SCF stimulation the cells were incubated with neutralizing antiCXCR4 antibody (Fig. 3C).

In another experiment, we used as the initial composition a sorted population of primitive $CD34+/CD38^{-/low}$ $CXCR4^{-/low}$. In order to obtain the initial cell composition, we first sorted out $CD34+/CD38^{-/low}$ cells from cord blood cells with a Fluorescence Activated Cell Sorter (FACS), and then we isolated non migrating $CD34+/CD38^{-/low}$ $CXCR4^{-/low}$ cells employing an in-vitro transwell assay. In this assay the FACS sorted $CD34+/CD38^{-/low}$ cells were placed in an upper chamber and SDF-1 in a lower chamber. With time, $CD34+/CD38^{-/low}$ cells with functional CXCR4 migrated to the SDF-1 in the lower chamber, while cells lacking functional CXCR4 did not migrate to SDF-1 and remained in the upper chamber. We collected the non-migrating $CD34+/CD38^{-/low}$ $CXCR4^{-/low}$ cells from the upper chamber and showed in the animal model that said non-migrating cells failed to engraft to the BM (Fig. 4A NM). We showed that subjecting said primitive non-migrating cell composition to

the SCF-treatment, restored the migratory capacity of the cells toward SDF-1 (reflecting increased CXCR4 expression) and engraftment potential of said cells (FIG. 4A, +SCF).

These results clearly demonstrate that restoring migration and engraftment potential in a population comprising mostly primitive non migrating human CD34+ $CD38^{-/low}$ $CXCR4^{-/low}$ cells by the short-term SCF stimulation cannot be explained by cell expansion, but only by a change in the phenotypic characteristics of the initial cell composition.

Thus, in all, the above experiments demonstrate that the first step of our method, namely increasing surface CXCR4 expression in the initial human primitive stem cells, increases the engraftment potential of said cells.The second step of our method ensures obtaining, among the expressing CXCR4 cells, those cells expressing functional CXCR4.

The self-renewal capability of a stem cell population *in vivo* is not trivial and it is crucial for the continuous supply of both myeloid and lymphoid cells in transplanted recipients. Self-renewal of stem cells can only be determined by the ability of the stem cell to repopulate secondary transplanted recipients with high numbers of both myeloid and lymphoid cells. We monitored the level of self renewal of a human stem cell composition by injecting bone marrow cells from mice engrafted with human cord blood (CB)

CD34+ cells in a secondary host and detecting the level of engrafted human CD34+ cells in the bone marrow of the secondary host. We observed little engraftment of secondary transplanted mice receiving human cells (Fig. 4B, panel a, 1st vs. 2nd) indicating that, the self-renewal capacity of secondary transplanted human stem cells is low. However, to our surprise, we found that if we subjected the bone marrow cells from engrafted mice to a short-term SCF/IL-6-stimulation prior to transplantation, we induced surface cell CXCR4 expression (Fig 4B, panel c), increased migration to SDF-1 in vitro (Fig. 4B, panel d), and enhanced engraftment of 2nd transplanted cells to a level which was comparable to that shown in 1st transplanted cells (Fig. 4B, panel b).

Thus, our method allows preparation of a cell composition comprising pluripotent stem cells with a surprising increased engraftment potential and enhanced self-renewal capacity in vivo.

In addition, three post filing published articles having supporting data showing unexpected results with other suitable stimulating agent such as low O2 (hypoxia) are attached hereto.

Schioppa et al., J. Exp. Med. 198(9):1391-1402(2003), shows that low O2, is an agent capable of inducing high expression of CXCR4 in different cell types.

Danet et al., J. Clin. Invest. 112(1):126-135 (2003), shows that bone marrow CD34+ CD38- cells cultured under low O2 conditions have increased repopulation activity which is associated with preferential expansion of CD34+CD38- cells. Danet et al states, "the use of low O2 levels to enhance the survival and/or self-renewal of human hematopoietic stem cells in vitro represents an important advance and could have valuable implications"

Ceradini et al., Nature Medicine 10(8):858-864(2004) shows that the recruitment of CXCR4 positive progenitor cells to site of injury is mediated by hypoxic gradients and is inhibited by blocking CXCR4 on circulating cells.

Regarding the disclosures and teachings of the cited and applied prior art references, Kanz (Kanz et al., U.S. Patent No. 5,541,103) starts with an initial composition of human $CD34^+$ cells and simply expands them. The ending material is merely a larger quantity of human $CD34^+$ cells.

The method of Kanz, involves culturing (not stimulating) a human CD34+ cell sample with a combination of cytokines and hematopoietic growth factors consisting essentially of interleukin-1 (IL-1), interleukin-3 (IL-3), interleukin-6 (IL-6), erythropoietin (EPO), and stem cell growth factor (SCF)(see claim 1). In fact, Kanz et al. does not show any cell expansion in human CD34+ cells cultured with

SCF alone (Kanz et al., U.S. Patent No. 5,541,103 Fig. 2) or with SCF and IL-6. Kanz shows dramatic expansion of human CD34+ cells only when human CD34+ cells are cultured with a combination of cytokines and hematopoietic growth factors consisting of, in addition to IL6 and SCF, other factors such as IL-3, IL-1 and EPO (Fig. 2).

Kanz is totally silent about the CXCR4 status, and self-renewal potential of pluripotent cells in vivo of the expanded cells, or of the non-expanded cells treated with SCF. No one of ordinary skill in the art reading Kanz et al. would expect that any characteristics of the cells such as surface CXCR4+, self-renewal capacity of primitive and pluripotent cell in vivo, and their engraftment potential, are being changed by the cell expansion method taught by Kanz or by treatment with SCF disclosed by Kanz. Thus, if 25% of the starting cells happen to be $CXCR4^{+}$, one would expect that 25% of the ending cells would be $CXCR4^{+}$.

I would also like to emphasize that our method for the preparation of a cell composition is different from the method of Kanz et al. For example, as we mentioned before, our method comprises stimulating a population comprising human CD34+ $CD38^{-/low}$ cells only for a short period of time (up to 5 days) in the presence of an agent capable of converting human CD34+ $CD38^{-/low}$ $CXCR4^{-/low}$ into a human CD34+ $CD38^{-/low}$ $CXCR4^{+}$ cell,

while Kanz's method teaches culturing human CD34+ cells with the combination of IL6, SCF, IL-3, IL-1 and EPO (Fig. 2) for longer periods of time to allow cell expansion (around 28 days, US 5,541,103 example 4 and 5).

Because our method and Kanz's method are different, the characteristics of the cell composition obtained by either method must be the different. For example, as mentioned, Kanz expands cells and obtains more of the same initial cell composition while we obtain a cell composition with different phenotypic characteristics from that of the initial cell composition.

Kanz et al. only discloses that SCF alone does not expand cells but does not show the elevation of CXCR4 or the positive effects of this CXCR4 elevation. Kanz teaches only expanded cells obtained with the combination of cytokines which allowed expansion (see US 5,541,103 Fig. 2) but not non-expanded cells such as those cultured with SCF alone. Therefore, not only is Kanz's method different from our method but Kanz also teaches away of our method.

Mohle et al., Blood 91(12): 4523-4530 (1998), merely teaches a technique for sorting out $CXCR4^+$ progenitor cells from an initial population of human CD34+ cells in vitro (page 4526 column 1). Since stem cells are identified by in vivo functional assays, which require homing and repopulation,

Mohle has no evidence for stem cells in the sorted CD34+ $CXCR4^+$ cells, because the work of Mohle is in vitro. For example, Ara et al., Immunity 19:257-267 (2003), a copy of which is attached hereto, revealed that expressing SDF-1 in the endothelium of SDF-1 knock out mice, stem cells but not committed progenitor cells, can home to the bone marrow (thus, the motility properties of stem cells and progenitors are not identical).

As explained above only a very low fraction of CD34+ cells (up to 20% of all cord blood CD34+ cells) are of more primitive type and of that fraction only 1/3 is of the type human $CD34+CD38^{-/low}$ $CXCR^+$. Thus, merely sorting out CD34+ $CXCR4^+$ cells from peripheral blood according to the method of Mohle may yield an insufficient amount of pluripotent stem cells. In addition, as mentioned above, Mohle has no evidence for stem cells in the sorted CD34+ $CXCR4^+$ cells.

Mohle does not suggest or motivate one of ordinary skill in the art to sort out human CXCR4+ cells starting from an initial composition of human CD34+ cells that were treated with an agent that increases CXCR4 expression in primitive stem cells, in order to obtain stem cells having the surprising feature of self-cell renewal in vivo.

There is nothing in Kanz to motivate one of ordinary skill in the art to use the sorting method of Mohle

with cells treated with an agent that increases surface CXCR4 expression. At most, Kanz may motivate the ordinary skill to use the sorting method of Mohle with an initial cell composition of expanded CD34+ cells. Moreover, Kanz teaches away from using in the sorting method of Mohle cells treated with SCF alone because this agent failed to induce expansion.

There is no reason for any one of ordinary skill in the art reading Kanz and Mohle to believe that a cell composition suitable for engraftment comprising more primitive and pluripotent cells capable of self renewal in vivo can be obtained by stimulating a cell composition comprising primitive cells with a suitable agent capable of converting said primitive cells from $CXCR4^-$ into CXCR4+, and by selecting for cells having functionally active CXCR4.

Consequently, the presently claimed invention, which demonstrates the above unexpectedly superior results, cannot be made obvious by the disclosures and teachings of Kanz and Mohle.

The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section

1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

May 16, 05
Date

צבי לפידות
Tsvee LAPIDOT

G:\BN\B\BENA\Lapidot2\pto\declaration 1.132.doc

ZX

CURRICULUM VITAE May 2005

A: Personal details:

Name: Tsvee Lapidot

Address: Department of Immunology, Weizmann Institute of Science
Rehovot 76100. Israel

Telephone: Tel: 972- 8-9342481 Fax: 972-8-9344141

Home Page: http://www.weizmann.ac.il/immunology/LapidotPage.html

Citizenship: Israeli

Marital status: Married + three children

B. Education:

1990-1994. Post-Doc. Hospital for Sick Children, University of Toronto.
Title: Animal Models for Normal and Leukemic Human Hematopoiesis.
Supervisor: Prof. John Dick

1985-1990. Ph.D. Weizmann Institute, Rehovot.
Title: T cell -Depleted Bone Marrow Transplantation in Mice: Mechanisms of Allograft Rejection and Enhancement.
Supervisor: Prof. Yair Reisner.

1983-1985. M.Sc. Weizmann Institute, Rehovot.
Title: The Role of Auto-Immune T Cells in Rheumatoid Arthritis.
Supervisor: Prof. Irun Cohen.

1980-1983. B.Sc. The Hebrew University. Jerusalem.

C. Employment:

2001-Present. Associate Professor, Department of Immunology, Weizmann Institute.

1994-2001. Senior Scientist. Department of Immunology. Weizmann Institute.

E. Other Appointments:

Past and Present Memberships:
Editorial Board of:
Experimental Hematology.
Stem Cells.
Stem Cell Reviews.
Stem Cells and Development.
Haplo.Org, website.

The scientific board of:
The 31st and 32nd annual meeting of the International Society of Experimental Hematology.
The American Society of Hematology (ASH) annual meeting, Scientific Committee on Hematopoietic Growth Factors.
Experimental Research Committee of the Israeli Society of Hematology.
The European Science Foundation review panel, on stem cells (EuroSTELLS 2004-5).
The organizing committee of the 2005 European community ESH-EBMT Euro-conference on stem cell research.
The Israel Science Foundation (ISF) review panel.

Teaching experience:
I have taught multiple courses on stem cell biology and hematopoiesis at the Weizmann Institute.

F. International recognition:

Fellowships and Awards:

August 1990-1992. European Molecular Biology Organization (EMBO) Long-Term Fellowship.

July 1992-1994. National Cancer Institute of Canada Post-doctoral Fellowship (NCIC).

July 1992. K.M Hunter Award for First Overall Ranked Fellowship Application (NCIC).

June 1993. Andrew Sass Kortsak Award for Scientific Achievements on Normal and Leukemic Human Hematopoiesis, The Hospital for Sick Children (HSC).

October 1994-1997. The Alon Fellowship for Top Ranked Young Israeli Scientists. The Israeli Government.

September 1994-1999. A Research Career Development Award (RCDA) for Young Israeli Scientists. Israel Cancer Research Fund (ICRF).

December 95. The L. Naftali Science Foundation for Biology and Medicine Award for young Israeli scientists.

February 96 – Present. The Pauline Recanati Career Development Chair of Immunology.

May 1999. The M. Levinson Award in Biology for Research on Mechanisms of Human Stem

Cell Migration and Function, Weizmann Institute.

May 2002. The James Heineman Research Award in Life Sciences. The Minna-James-Heineman Foundation. Munich, Germany.

Review of Grants and Manuscripts:

Scientific grants and scholarship stature for academic promotion from Europe, North America, Australia and Israel were reviewed.

Review of manuscripts in the fields of human and murine hematopoietic stem cell biology, both normal and leukemic. The roles of, cytokines, proteolytic enzymes, adhesion molecules and chemokines, in particular SDF-1/CXCR4 interactions in stem cell homing, development and mobilization.

Requests for manuscript review were received from the following journals:

AJH,	PloS Biology,	Immunity,
Blood,	PNAS,	J of Leukocyte Biology,
BBA,	Transplantation,	Leukemia,
Biochemistry,	J. of Clinical Investigation,	Nature,
Biotechniques,	J. of Exp. Medicine,	Nature Immunology,
Bone Marrow Transp.	Experimental Hematology,	Nature Medicine,
Circulation,	E. J. of Immunology,	Science,
Gene Therapy,	E. J. of Hematology,	Stem Cells.

Invited lectures at Scientific Centers and National Meetings (last 5 years):

11. June 5-7 2000 University of Lund, Sweden. Title: Mechanism of Human Stem Cell Migration and Development.

12. July 19 2000. M.D Anderson, Houston, Texas, U.S.A. Title: Mechanism of Human Stem Cell Migration and Function.

13. October 12, 2000 Hopital Gustav Rousi VilleJuif, Paris, France. Title: Mechanism of human stem cell migration and function in transplanted NOD/SCID mice.

14. November 28, 2000 Novartis, Vienna, Austria. Title: Mechanism of human stem cell migration and function.

15. November 30, 2000 Mount-Sinai New York, U.S.A. Title: Mechanism of human stem cell migration and function in transplanted NOD/SCID mice.

16. February 5, 2001 The 30ND Annual Meeting, The Israeli Immunology Society, The Weizmann Institute. Title: The essential roles of SDF-1/CXCR4 interactions in human stem cell homing and repopulation of transplanted NOD/SCID mice.

17. February 17, 2001, European Network for Fetal Transplantation (ENFET) Launch Meeting,

Nottingham, UK. Title: Mechanism of Human Stem Cell Homing and repopulation in Transplanted NOD/SCID mice.

18. May 2, 2001 Millennium Pharmaceutical, Inflammation branch Boston MA, U.S.A. Title: Mechanism of human stem cell migration and repopulation in transplanted immune deficient mice.

19. May 3, 2001 NIH NHLB, Bethesda MD, U.S.A. Title: Mechanism of human stem cell migration and repopulation in transplanted immune deficient mice.

20. May 4, 2001 Rochester University, NY, U.S.A. Title: The role of SDF-1/CXCR4 interactions in human stem cell migration and repopulation of NOD/SCID mice.

21. June 19. 2001 Montpellier University, Montpellier , France. Title: Mechanism of human stem cell T lymphocyte development in transplanted NOD/SCID mice in response to TNF-a.

22. September 10, 2001 The Hospital for Sick Children, Toronto, Canada: Mechanism of human stem cell migration and function in transplanted NOD/SCID mice: role of SDF-1/CXCR4 interactions.

23. November 26, 2001 Institute for Cancer Research IRCC, Torino, Italy: Mechanism of human stem cell homing and mobilization: The role of proteolytic enzymes, SDF-1 degradation and increased CXCR4 expression.

24. January 13-15, 2002, Eilat Israel. France Israel AFIRST meeting. Title: Induction of human T cell development by SRC/stem cells in the BM and spleen of NOD/SCID mice by TNF-α.

25. February 4 – 7, 2002. Eilat Israel. The Israeli FISEB/ILANIT meeting. Ttile: Sorted human cord blood CD34+CXCR4- cells contain internal CXCR4 which can oscillate in vivo and rescue low SDF-1 dependent repopulation in NOD/SCID mice

26. September 13th 2002 Institute Cochin, Paris, France, XIXth Journée Jean-Claude Dreyfus, STEM CELLS : Differentiation and Plasticity . Title: The essential roles of SDF-1/CXCR4 interactions in human stem cell homing and mobilization in transplanted NOD/SCID mice

27. Nov. 15th 2002. Euro Cord III, Paris , France. Title: The essential roles of SDF-1/CXCR4 interactions in human stem cell homing and mobilization in transplanted NOD/SCID mice.

28. Nov.28-29 2002 Stem Cell Potentials, Rotterdam, Holland. Title: The role of SDF-1/CXCR4 interactions in normal and leukemic human stem cell motility.

29. February 12-13, 2003 The 32ND Annual Meeting, The Israeli Immunology Society, Technion, Haifa. Israel Title: The essential roles of SDF-1/CXCR4 interactions in human stem cell homing and repopulation of transplanted NOD/SCID mice.

30. March 6, 2003. Rabin Medical Center , Petach-Tikva. Israel. Ttile: The roles of SDF-1/CXCR4 interactions in normal and leukemic human stem cell homing and repopulation of NOD/SCID mice.

31. April 3 –20 2003, invited to give 3 talks in Australia at Westmead Institute for Cancer research Sydney, Stem Cell Laboratory, Peter MacCallum Cancer Insititute Melbourne and the Institute of Medical Science Adelaide South Australia. Title: The essential roles of SDF-1/CXCR4 interactions in human stem cell migration and development in transplanted NOD/SCID mice.

32. April 28, 20003 ProChon and April 1 BTG, Scientific Park, Nes-Ziona, Israel. Ttile: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human stem cell migration and development.

33. June 8, 2003, Dept. of Biology The Technion, Haifa Israel. Title: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human stem cell migration and development.

34. June 23, 2003, 2 Chemokine Day, Tel-Aviv university, Israel. Title: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human stem cell migration and development.

35. June 29-30, 2003, Weizmann Institute, France/Israel Workshop on Stem Cells. Ttile: Stem cell migration, adhesion and homing.

36. September 21, First Frankel Conference on Stem Cell Biology, Schneider Childrens medical Center, Petach-Tikva, Israel. Title: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human hematopoiesis in transplanted NOD/SCID mice.

37. November 18, 20003, Cancer Research Insitute, London, UK. Title: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human hematopoiesis in transplanted NOD/SCID mice.

38. December 5th, 2003 The Pre ASH Myeloid and Stem cell meeting, San – Diego, CA, U.S.A, Title: Regulation of stem cell migration to the injured liver in response to stress signals.

39. March 1st, 2004, Israeli MEDAX, Medicine meeting Tel-Aviv, Israel. Title: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human hematopoiesis in transplanted NOD/SCID mice.

40. May 10-13, 2004, Weizmann Institute joint Albert Einstein Weizmann meeting. Title: Hematopoietic stem cell recruitment to the liver in response to stress signals.

41. July 12, 2004, Rush Presbyterian, St. Lukes Medical Center, Chicago, Il. , U.S.A. Grand Rounds, Ttile: Current understanding of factors influencing normal and leukemic human stem cell migration and proliferation: the role of chemokines, cytokines proteolytic enzymes and adhesion molecules.

42. July 14, 2004, Univ. of Texas, Health Science Center, San-Antonio, Texas, U.S.A. Grand Rounds, Title: Current understanding of factors influencing normal and leukemic human stem cell migration and proliferation: the role of chemokines, cytokines proteolytic enzymes and adhesion molecules.

43. August 5, 2004. Sheba Medical Center, Opening of the new Hematology & Stem Cell Transplantation Center. Title: Stem Cell Mobilization-Molecular Pathways.

44. November 28 – 29, 2004. First International Stem cell Meeting. The Weizmann Institute. Title: Regulation of Human Stem Cell Migration and Development: Molecular pathways.

45. January 18, 2005. Louiville University, KY, U.S.A. Title: Mechanism of human stem cell homing and mobilization, the role of: Ocl./Ob., SDF-1/CXCR4, MT1-MMP and CD45.

46. January 25, 2005. NIH, Bethesda MD, US.A. Title: Mechanism of human stem cell homing and mobilization, the role of: Ocl./Ob., SDF-1/CXCR4, MT1-MMP and CD45.

47. February 1, 2005 Tel-Aviv. The 39th Israel Society for Laboratory Medicine Meeting. Title; Regulation of human stem cell homing and repopulation in transplanted NOD/SCID mice

48. February 7-10, 2005 Eilat. Federation of Israeli societies for experimental biology (FISEB). Title: Regulation of human stem cell homing and repopulation in transplanted NOD/SCID mice.

49. February 16, 2005 ben-Gurion U, Beer-Sheba. Israeli Immunology society meeting. Title: Mechanism of human stem cell migration in transplanted NOD/SCID mice.

50. February 22, 2005. Tel – Aviv University. Title: Mechanism of human stem cell migration in transplanted NOD/SCID mice.

51. May 18th, 2005. Bar-Ilan University. Title: Regulation of human CD34+ stem cell migration and development in immune deficient NOD/SCID mice.

Invited Speaker in International Scientific Meetings (last 5 years):

11. April 9-11 2000, University of Munster, Germany. Transplantation in Hematology and Oncology II Meeting: Title: Dependence of Human Stem Cell Engraftment of NOD/SCID Mice on CXCR4.

12. July 14-18 2000. New York Medical College,: 13th International Symposium on Molecular Biology of Hematopoiesis, NY, U.S.A. Title: Mechanism of Human Stem Cell Migration and Function.

13. September 10-14 2000 Male-Hamisha Israel. International Meeting on Inflammatory Cytokines and Chemokines in the Context of Extracellular Matrix. Title: Mechanism of Human Stem Cell Migration and Function.

14. September 14-16 2000, University of Tubingen, Germany: 3rd International Conference and Workshop on Hematopoietic Stem Cells. Title: Mechanism of Human Stem Cell Migration and Function.

15. October 5-7 2000, XV Congress of The Italian Cancer Society, Torino, Italy. Title: Mechanism of Human Stem Cell Migration and Function.

16. April 30- May 1, 2001, Third International Indianapolis Conference/Workshop: Recent Developments in Cord Blood Stem and Progenitor Cell Transplantation and Biology, Indiana, U.S.A. Title: Mechanism of Human Stem Cell Homing and Repopulation in Transplanted NOD/SCID and B2mnull NOD/SCID mice.

17. June 21-24, 2001. Frankfurt Germany. The 6th meeting of the European Hematology Association. Title: Mechanism of Human Stem Cell Homing and Repopulation in Transplanted NOD/SCID and B2mnull NOD/SCID mice.

18. June 6-9, 2002, Florence, Italy The 7th meeting of the European Hematology Association. Title: Mechanism of Human Stem Cell Homing and Repopulation in Transplanted NOD/SCID and B2mnull NOD/SCID mice.

19. June 28 – 29, 2002. Orvieto, Italy. 3rd European Workshop on Haploidentical Stem Cell Transplantation. Title: Induction of human T cell development in transplanted NOD/SCID mice by TNFα and TGFβ.

20. September 6-7, 2002, Munster Germany, 3rd Transplantation in Hematology and Oncology Meeting. Key Note Lecture. Title: Stem Cell Mobility and Homing.

21. February 13-15, 2003. Paris France, ESH meeting on Hematopoietic Stem Cell Biology and Transplantation. Title: Cell Migration, Adhesion and Stem cell Homing.

22. July 20-23, 2003, Istanbul Turkey, 29th EBMT meeting. Plenary session on Stem Cell Biology. Title: Stem Cell Homing.

23. August 27-30, 2003, Curitiba Brazil. The XVIII annual meeting of the Brazilian Societies of Experimental Biology (Reuniao Anual da Federaçao de Sociedades de Biologia Experimental, (FESBE)). Title: Mechanism of human stem cell migration in transplanted NOD/SCID mice: the role of chemokines, cytokines, adhesion molecules and proteolytic enzymes

24. November 20-21, Grenoble France, 20th Normal and Leukemic Hematopoiesis Meeting, French Hematology and BMT Societies. Title: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human hematopoiesis in transplanted NOD/SCID mice.

25. December 5-9, 2003, San-Diego U.S.A, 45th meeting of The American Society of Hematology (ASH), The Education Session on Stem Cell Mobilization. Title: Current understanding of factors influencing stem cell mobilization.

26. March 21-23, Bologna, Italy. Adult Stem Cells Meeting. Title: Stress signals mediate migration and engraftment of hematopoietic stem cells into non hematapoietic tissues.

27. June 7-9, 2004, Cracow Poland, 2nd Annual Stem Cell Excellence Centre Meeting (STEC). Title: The essential roles of SDF-1/CXCR4 interactions in normal and leukemic human hematopoiesis in transplanted NOD/SCID mice.

28. June 17-18, Mira, Italy, Blood and Marrow Stem Cell Transplantation in Hematology and Oncology meeting. Title: Hematopoietic Stem Cell Biology.

29. July 17-20, 2004, New Orleans U.S.A, Annual meeting of the International Society of Experimental Hematology (ISEH), Plenary Session : Stem Cell biology. Title: Mechanisms of stem cell homing and mobilization.

30. October 21-23 2004, Heidelberg Germany. International Cytometry Meeting of the German Cancer Society. Stem cell session. Title: Mechanism of human stem cell homing and repopulation in transplanted NOD/SCID mice.

31. November 19-21, 2004, Adelaide Australia, Hanson Symposium on Stem Cell Mobilization. Title: Current understanding of factors influencing stem cell mobilization.

32. January 20 – 22, 2005, Cancun Mexico. 5th International Workshop on Stem Cell Transplantation. Stem Cell Mobilization Session. Title: Mechanism of Stem Cell Mobilization: The role of Ocl./Obl., SDF-1, HGF, MT1-MMP and CD45.

33. April 15-17, 2005. Cascais, Portugal. ESH- EBMT Euro-conference on stem cell research. Title: Mechanism of human stem cell homing and mobilization in transplanted NOD/SCID mice.

34. June 18-22, 2005. Wilsede, Germany. XVI Wilsede Meeting, Modern Trends in Human Leukemia. Stem cell session. Title: Mechanism of human stem cell homing and mobilization in transplanted NOD/SCID mice.

35. October 7-9, 2005, Cracow Poland. 3nd Annual Stem Cell Excellence Centre Meeting. (STEC). Keynote lecture: Mechanism of human stem cell migration to and from the bone marrow.

G. Scientific productivity:

Grants (last 5 years):

Year	**Title**	**Agency**	**Amount/ Year ($)**
01/01/05 - 31/12/05	Animal models for human stem cells.	Pluristem	36,432
Approved, in process	The role of neutrophil elastase in the development and invasiveness of human AML stem cells	Weizmann/ Ichilov	50,000
01/12/04 - 30/11/05	Human stem cell biology.	Meller Center	1,306
01/12/03 - 30/11/04	Regulation of human stem cell migration.	Schnur Ms. Silvia	10,000
01/09/03 - 31/08/04	Migration and development of human leukemic AML stem cells	Goldenson I.&L.	20,000
01/10/04 - 30/09/08	Regulation of the bone marrow reservoir in response to stress signals	ISF	54,175

Year	Title	Agency	Amount/ Year ($)
01/10/03 - 30/09/04	SDF-1-mediated adhesion and motility of normal and malignant human stem cells	Center for Scientific Excellence	20.000
01/01/04 - 31/12/05	SDF-1/CXCR4 signaling pathways in human precursor-B acute lymphoblastic leukemia (pre-B ALL) cell migration, invasion, survival and proliferation	ICA	8,478
01/05/02 – 28/2/03. 1/9/04 - 31/08/05.	Animal models for human stem cells.	Gamidacell, IL	42,504
01/06/02 - 31/05/03	Award for first over all rated scientist among tenure applicants at Weizmann, 2000.	Heineman James Research Award	22,845
01/03/03 - 28/02/06	Evaluation of the facilitation of umbilical cord blood transplant engrafment using ex vivo haematopoietic progenitor/stem cell expansion	EU / FP5 / QOL - Quality of Life	9,343
01/01/02 - 31/12/04	The role of SDF-1 signaling in human and murine stem cell migration in transplanted immune deficient mice	Minerva	55,740
01/10/01 - 30/09/05	Regulation of normal and leukemic human AML stem cells.	Rich Foundation	59,875
01/07/01 - 30/06/02	Animal models for human stem cells.	Gamidacell, IL	41,400
01/06/01 - 31/05/02	Stem cell migration to the liver.	Levine Center for Applied Research	30,000
01/12/00 - 30/11/04	European network for fetal transplantation	EU / FP5 / QOL - Quality of Life	10,631
01/10/00 - 30/09/03	T-cell differentiation potential of genetically modified stem cells propagated on stromal cell lines: Model of human zap-70 deficiency	Afirst Israel/France	43,906
01/07/00 - 30/06/02	migration patterns of normal and leukimic stem cells from children	Concern Foundation	50,000
01/09/00 - 31/08/02	Human CML stem cells	ICRF - PG	20,000
01/10/00 - 30/09/04	The mechanisms of human stem and progenitor cells homing to the bone marrow, their retention, release, or mobilization into the blood	ISF	45,220
01/10/99 - 30/09/02	Stormal cell-associated maintenance or expansion of transplantable human haemopoietic stem cells in threedimensional cultures	ISF	10,000
01/04/98 - 30/06/05	Clinical models for human stem cell migration and development.	Ares Serono	131,556

Past and current students:

M.Sc.:	Year:	Current activity:
Ifat Fajerman	1996-1998	Post Doc with Nobel laureate, Ciechanover A.
Michal Magid	1999-2000	PhD in Philadelphia, USA.
Asaf Spiegel	2000-2001	PhD in our lab
Ph.D:		
Orit Kollet	1995-1999	Staff Scientist in our lab
Isabelle Petit	1998-2002	Post Doc in Cornell, NY, USA, Supervisor: Rafii S.
Tanya Ponomaryov	1998-2003	Post Doc in Weizmann, Supervisor: Levkowitz, G.
Sarit Samira	2000-2004	Biotech company.
Joy Kahn	2001-2005	Post Doc in Weizmann, Supervisor: Zelzer E.
Ayelet Dar	2001-2005	PhD in our lab.
Asaf Spiegel	2001-2005	PhD in our lab.
Shoham Shivtiel	2002-2006	PhD in our lab.
Post Doctoral fellows:		
Amnon Peled	1998-2000	Lab head in Hebrew U., Haddassah Medical Center.
Tamara Byk	1999-2000	Biotech company.
BienLing Liu	1999-2000	Post Doc in Indiana, USA. Supervisor: van Der Loo JC.
Orit Kollet	2000-2001	Staff Scientist in our lab.
Abraham Avigdor	2002-2003	M.D. Sheba Medical Center.
Polina Goichberg	2002-2004	Staff Scientist in our lab.
Sigal Tavor	2002 –2003	M.D. Sourasky Medical Center.
Isabelle Petit	2003-2005	Post Doc in Cornell, NY, USA, Supervisor: Rafii S.
Svetlana Porozov	2004	Biotech company.
Yifat Levy	2005-2006	
Scientists:		
Orit Kollet	2001-present	
Alexander Kalinkovich	2002-present	
Polina Goichberg	2005-present	
Technician:		
Loya Abel	2001-2003	(retired)

National and International collaborations (major):

National: Alon R., Zipori D., Ravel M., Nagler A. Canaani E., Wallach D., Naor D.

International: Piacibello W., Shafritz D., Roit A., Fujii N., Teichamn R., Kaarlson S., Arenzana-Seisdedos F., Bik To L., Taylor N.

H. Patents:

1. Yeda's Ref.: **9744**
Title: *Chimeric interleukin-6 soluble receptor/ligand protein, analogs thereof and uses thereof*
Inventors: Michel Revel, Judith Chebath, Tsvee Lapidot, Orit Kollet
Patents: Australia – No. 758161; Eurasia – No. 004793; New Zealand – No. 502139; South Africa – No. 98/6145

2. Yeda's Ref.: **9859**
Title: *Hematopoietic cell composition for use in transplantation*
Inventors: Tsvee Lapidot, Amnon Peled
Application: PCT patent application – Publication No.: WO 00/06704

3. Yeda's Ref.: **2001-119**
Title: *Migration of haematopoietic stem cells and progenitor cells to the liver*
Inventors: Orit Kollet, Tsvee Lapidot
Application: PCT patent application – Publication No.: WO 03/047616

4. Yeda's Ref.: **2003-048**
Title: *Stem cells having increased sensitivity to a chemoattractant and methods of generating and using same*
Inventors: Orit Kollet, Tsvee Lapidot
Application: PCT patent application – Publication No.: WO 2004/090120

5. Yeda's Ref.: **2003-049**
Title: *Stem cells having increased sensitivity to a chemoattractant and methods of generating and using same*
Inventors: Orit Kollet, Tsvee Lapidot
Application: PCT patent application – Publication No.: WO 2004/090121

6. Yeda's Ref.: **2003-101**
Title: *Methods of generating and using stem cells enriched with immature primitive progenitor*
Inventors: Orit Kollet, Tsvee Lapidot
Application: PCT patent application No. PCT/IL2004/001018 (pre-publication)

7. Yeda's Ref.: **2004-057**
Title: *Elastase inhibitor and acute leukemia*
Inventors: Tsvee Lapidot, Sigal Tavor, Isabelle Petit
Application: Israel patent application No. 163453 (filed 10.08.2004)

8. Yeda's Ref.: **2004-065**
Title: *The use of a protease or a protease inhibitor for the manufacture of medicaments*
Inventors: Orit Kollet, Tsvee Lapidot
Application: Israel patent application No. 164942 (filed 31.10.2004)

I. Languages:

Fluent reading, writing and speaking of Hebrew and English.

Tsvee Lapidot, List of Publications:

Refereed articles:

1. Holoshitz J. Drucker I. Yaretzky A. Van Eden W. Klajman A. **Lapidot T.** Frenkel A. & Cohen IR. (1986). T lymphocytes of rheumatoid arthritis patients show augmented reactivity to a fraction of mycobacteria cross-reactive with cartilage. **Lancet** ii: 305-309.

2. Schwartz E. **Lapidot T.** Gozes D. Singer TS. & Reisner Y. (1987). Abrogation of bone marrow allograft resistance in mice by increased total body irradiation correlates with eradication of host clonable T cells and alloreactive cytotoxic precursors. **J. Immunology.** 138: 460-465.

3. **Lapidot T.** Singer TS. & Reisner Y. (1988). Transient engraftment of T cell depleted allogeneic bone marrow in mice improves survival rate following lethal irradiation. **Bone Marrow Transplantation.** 3: 157-164.

4. **Lapidot T.** Singer TS. Schwartz E. & Reisner Y. (1988). Booster irradiation to the spleen following total body irradiation: a new immunosuppressive approach for allogeneic bone marrow transplantation. **J. Immunology.** 141: 2614-2619.

5. **Lapidot T.** Terenzi A. Singer TS. Salomon O. & Reisner Y. (1989). Enhancement by dimethyl myleran of donor type chimerism in murine recipients of bone marrow allografts. **Blood** 73: 2025-2032.

6. **Lapidot T.** Lubin I. Faktorowich Y. Erlich P. & Reisner, Y. (1990). Enhancement of bone marrow allografts from "nude" mice into mismatched recipients by T cells void of graft versus host activity. **PNAS.** USA 87: 4595-4599.

7. Salomon O. **Lapidot T.** Terenzi A. Lubin I. Rabi I. & Reisner, Y.(1990). Induction of donor-type chimerism in murine recipients of bone marrow allografts by different radiation regimens currently used in treatment of leukemia patients. **Blood** 76: 1872-1878.

8. Terenzi A. Lubin I. **Lapidot T.** Salomon O. Faktorowich Y. Rabi I. Martelli MF. & Reisner Y. (1990). Enhancement of T cell depleted bone marrow allografts in mice by Thiotepa. **Transplantation.** 50: 717-720.

9. Lubin I. Faktorowich I. **Lapidot T.** Gan Y. Eshhar Z. Gazit E. Levite M. & Reisner Y. (1991). Engraftment and development of human T and B cells in mice after bone marrow transplantation. **Science** 252: 427-431.

10. **Lapidot T.** Facktorovich Y. Lubin I. & Reisner Y. (1992). Enhancement of T cell depleted bone marrow allografts in the absence of Graft Versus Host Disease is mediated by $CD8^+$ $CD4^-$ and not by $CD8^-CD4^+$ thymocytes. **Blood** 80: 2406-2411.

11. Facktorovich Y. **Lapidot T.** Lubin I. Reisner Y. (1993). Enhancement of BM allografting from C57BL/6 'nude' mice into C3H/HEJ recipients by tolerized T cells from (C57BL/6 -> C3H) and (C3H/HEJ -> C57BL/6) chimeras. **Bone Marrow Transplantation** 12: 15 –20.

12. Weiss L. Lubin I. Factorowich I. **Lapidot T**. Reich S. Reisner Y. & Slavin S. (1994). Effective Graft Versus Leukemia effects independent of Graft Versus Host Disease after T cell depleted allogeneic bone marrow transplantation in a murine model of B cell Leukemia/Lymphoma. **J. Immunology** 153: 2562-2567.

13.Kamel-Reid S. Letarte M. Doedens M. Greaves A. Murdoch B. Grunberger T. **Lapidot T**. Thorner P. Freedman M. Philips R. & Dick J.(1991). Bone marrow from children in relapse with Pre-B acute lymphoblastic leukemia proliferates and disseminates rapidly in SCID mice. **Blood** 78: 2973-2981.

14. **Lapidot T**. Pflumio F. Doedens M. Murdoch B. Williams D. & Dick J. (1992). Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. **Science** 255: 1137-1141.

15.Pflumio F. **Lapidot T**. Murdoch B. Patterson B. & Dick J. (1993). Engraftment of human lymphoid cells into newborn SCID mice leads to Graft versus Host disease. **I. Immunology** 5,12: 1509-1522.

16. **Lapidot T**. Sirard C, Vormoor J, Hoang T, Cortes J, Minden M, Paterson B, Caliguri M, and Dick J. (1994). A cell initiating human acute myeloid leukemia after transplantation into SCID mice. **Nature** 367: 645-648.

17. Vormoor J, **Lapidot T**. Pflumio F. Risdon G. Broxmyer H. & Dick J, (1994). Immature human cord blood cells give rise to multi lineage progenitors in SCID mice. **Blood**. 83: 2489 - 2497.

18. Larochelle A. Vormoor J. **Lapidot T**. Sher G. Furukawa T. Li Q. Schultz L. Olivieri N. Stamatoyannopoulos G. & Dick J. (1995). Engraftment of immune- deficient mice with primitive hematopoietic cells from β–Thalassemia and sickle cell anemia patients: implications for evaluating human gene therapy protocols. **Human Molecular Genetics**. 4: 163-172.

19. **Lapidot T**. Sirard C. Vormoor J. Cashman J. Doedens M. Murdoch B. Jamal N. Messner H. Addey L. Minden M. Laraya P. Keating A. Eaves A. Lansdorp P. Eaves C. & Dick J. (1996). Normal and leukemic SCID-repopulating cells (SRC) coexist in the bone marrow and peripheral blood from CML patients in chronic phase while leukemic SRC are detected in blast crisis. **Blood**. 87: 1539-48.

20. Meydan N. Grunberger T. Dadi H. Shahar M. Arpaia E. **Lapidot T**. Leader S. Freedman M. Cohen A. Gazit A. Levitzki A. & Roifman C. (1996). Inhibition of Acute Lymphoblastic Leukemia by a JAK-2 Inhibitor. **Nature**, 379: 645-648.

21. **Lapidot T.** Grunberger T. Vormoor J. Estrov Z. Kollet O. Bunin N. Zaizov R. Williams D. and Freedman M. (1996). Identification of Human Juvenile Chronic Myelogenous Leukemia Stem Cells Capable of Initiating the Disease in Primary and Secondary Transplanted SCID Mice. **Blood**, 88: 2655-2664.

22. Larochelle A. Vormoor J. Haneberg H. Wang J. Bhatia M. **Lapidot T.** Moritz T. Murdoch B. Xiao X. Kato I. Williams D.& Dick. J. (1996). Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy.

Nature Medicine 12: 1329-1337.

23. Haran-Ghera N. Krautghamer R. **Lapidot T.** Peled A. Domingues M. & Stanley E.R. (1997). Increased circulating CSF-1 in SJL/J mice with radiation induced AML is associated with autocrine regulation of AML cells by CSF-1. **Blood** 89,7: 2537-2545.

24. **Lapidot T.** Cashman J. Wang J. Doedens M. Schultz L. Lansdorp P. Dick J. & Eaves C. (1997). Kinetic evidence of the regeneration of multi-lineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. **Blood** 89:4307-4316.

25. Ariel A. Hershkovitz R. Cahalon L. Williams D. Akiyama S. Yamada K. Chen C. Alon R. **Lapidot T.** & Lider O. (1997). Induction of T cell adhesion to extracellular matrix or endothelial cell ligands by soluble or matrix bound Interleukin-7. **Eur. J. Immonology** 27 :2562-2570.

26. Chebath J. Fischer D. Kumar A. **Lapidot T.** Kollet O. Rose-John S. Nagler A. Slavin S. & Revel M. (1997). IL-6 receptor, IL-6 fusion proteins with enhanced IL-6 type pleiotropic activities. **Eur. Cytokine Network**, 4: 359-365.

27. **Lapidot T.** Wang J.C.Y. Cashman J.D. Doedens M. Addy L. Sutherland D.R. Nayar R. Laraya P. Minden M. Keating A. Eaves A.C. Eaves C.J. and Dick J.E. (1998). High Level Engraftment of NOD/SCID Mice by Primitive Normal and Leukemic Hematopoietic Cells from Patients With Chronic Myeloid Leukemia in Chronic Phase. **Blood** 91: 2406-2414.

28. Varfolomeev E. Schuchmann M. Luria V. Chiannilkulchai N. Beckmann J. Mett I. Rebrikov D. Brodianski V. Kemper O. Kollet O. **Lapidot T.** Soffer D. Sobe T. Avraham K. Goncharov T. Holtmann. H. Lonai P. & Wallach D. (1998). Targeted disruption of the mouse caspase-8 gene ablates cell-death induction by the TNF receptors Fas/Apol1 and DR3 and is lethal prenatally. **Immunity** 9(2): 267-276.

29. Globerson A. Kollet O. Abel L. Fajerman I. Ballin A. Nagler A. Slavin S. Ben-Hur H. Hagay Z. Sharp A. & **Lapidot T.** (1999) Differential effects of $CD4^+$ and $CD8^+$ cells on lymphocyte development from human cord blood cells in murine fetal thymus explants. **Exp. Hematology** 2:282-92.

30. Peled A. Petit I. Kollet O. Magid M. Ponomaryov T. Byk T. Nagler A. Ben-Hur H. Many A. Shultz L. Lider O. Alon R. Zipori D.& **Lapidot T.**(1999). Dependence of Human Stem Cell Engraftment and Repopulation of NOD/SCID Mice on CXCR4. **Science** 283: 845-848.

31. Piacibello W. Sanavio F. Severino A. Dane A. Gammaitoni L. Fagioli F. Perissinotto E. Cavalloni G. Kollet O. **Lapidot T.** and. Aglietta M. (1999). Engraftment of Nonobese Diabetic Severe Combined Immunodeficient Mice of Human $CD34^+$ Cord Blood Cells After Ex Vivo Expansion: Evidence for the Amplification and Self-Renewal of Repopulating Stem Cells. **Blood** 93, 11:3736-49.

32. Kollet O. Aviram R. Chebath J. Ben-Hur H. Nagler A. Schultz L. Revel M. & **Lapidot T.** (1999) The soluble IL-6 receptor/IL-6 fusion protein enhances maintenance and proliferation of human $CD34^+$ $CD38^{-/low}$/SCID repopulating cells (SRC) in vitro. **Blood** 94, 3:923-31.

33. Peled A. Grabovsky V. Habler L. Sandbank J. Arenzana-Seisdedos F. Petit I. Ben-Hur H. **Lapidot T.** & Alon R. (1999). The Chemokine SDF-1 Stimulates Integrin-Mediated Arrest of Immature Human $CD34^+$ cells on Vascular Endothelium Under Shear Flow. **J. Clinical Investigations.** 104:1199-1211.

34. Shultz LD, Lang PA, Christianson SW, Gott B, Lyons B, Umeda S, Leiter E, Hesselton R, Wagar EJ, Leif JH, Kollet O, **Lapidot T**,& Greiner DL. (2000). NOD/LtSz-Rag-1null mice: An immunodeficient and radioresistant model for engraftment of human hematolymphoid cells, HIV infection, and adoptive transfer of NOD mouse diabetogenic T cells. **J. Immunology** 164(5):2496-507.

35. Kollet O. Peled A. Byk T. Ben-Hur H. Greiner D. Schultz L. & **Lapidot T**. (2000). B2 Microglobulin Deficient ($B2m^{null}$) NOD/SCID Mice are Excellent Recipients for Studying Human Stem Cell Function. **Blood**. 95, 10:3102-5.

36. Peled A. Kollet O. Ponomaryov T. Petit I. Franitza S. Grabovsky V. Magid M. Nagler A. Lider O. Alon R. Zipori D. & **Lapidot T**. (2000). The chemokine SDF-1 activates the integrins LFA-1, VLA-4 and VLA-5 on immature human $CD34^+$ cells: role in transendothelial/stromal migration and engraftment of NOD/SCID Mice. **Blood** 95,11: 3289-96.

37. Kollet O. Moore J. Aviram R. Ben-Hur H. Liu B. Nagler A. Shultz L. Feldman M. & **Lapidot T.** (2000). The plant lectin FRIL supports prolonged in vitro maintenance of quiescent human cord blood $CD34^+/CD38^{-/low}$/SCID repopulating cells. **Exp. Hematol** 28, 6:726-36.

38. Grabovsky V. Feigelson S. Chen C. Bliejs D. Peled A. Cinamon G. Baleux F. Arenzana-Seisdedos F. **Lapidot T**. Van-Kooyk Y. Lobb R. & Alon R. (2000). Sub-second induction of $\alpha 4$ integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial VCAM-1 under flow conditions. **J. Exp. Med.**.192: 495-506.

39. Ponomaryov T. Peled A. Petit I. Taichman R. Habler L. Sandbank J. Arenzana-Seisdedos F. Magerus A. Caruz A. Fujii N. Nagler A. Lahav M. Szyper-Kravitz M. Zipori D. & **Lapidot T.**(2000). Induction of the Chemokine Stromal-Derived-Factor-1 Following DNA Damage Improves Human Stem Cell Function .**J. Clinical Investigations**. 106:133-9.

40. Böldicke T. Tesar M. Griesel C. Rohde M. Gröne HJ. Waltenberger J. Kollet O. **Lapidot T**. Yayon A. & Weich H. (2001). Single-chain antibodies recognizing the human vascular endothelial growth factor receptor-2 (VEGFR-2/flk-1) on the surface of primary endothelial cells and preselected $CD34^+$ cells from cord blood. **Stem Cells**. 19:24-36.

41. Kollet O. Spiegel A. Peled A. Petit I. Byk T. Hershkovitz R. Guetta E. Barkai G. Nagler A. & **Lapidot T.** (2001). Rapid and efficient homing of human $CD34^+CD38^{-/low}CXCR4^+$ stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/$B2m^{null}$ mice **Blood** 97, 10: 3283-91.

42. Franitza S, Kollet O, Brill A, Vaday GG, Petit I, **Lapidot T**, Alon R, & Lider O. (2002). TGF-beta enhances SDF-1alpha-induced chemotaxis and homing of naive T cells by up-regulating CXCR4 expression and downstream cytoskeletal effector molecules. **Eur J Immunol** 32:193-202

43. Peled A, Hardan I, Trakhtenbrot L, Gur E, Magid M, Darash-Yahana M, Cohen N, Grabovsky V, Franitza S, Kollet O, Lider O, Alon R, Rechavi G, & **Lapidot T.** (2002). Immature Leukemic CD34(+)CXCR4(+) Cells from CML Patients Have Lower Integrin-Dependent Migration and Adhesion in Response to the Chemokine SDF-1. **Stem Cells.**; 20(3):259-266.

44. Petit I, Szyper-Kravitz M, Nagler A, Lahav M, Peled A, Habler L, Ponomaryov T, Teichman R, Arenzana-Seisdedos F, Fujii N, Sandbank J, Zipori D, & **Lapidot T.** (2002). G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and upregulating CXCR4. **Nature Immunology**,3, 687–694.

45. Kollet O, Petit I, Kahn J, Samira S, Dar A, Peled A, Deutsch V, Gunetti M, Piacibello W, Nagler A, & **Lapidot T.** (2002). Human CD34 + /CXCR4 - sorted cells harbor intracellular CXCR4, which can functionally be expressed and provide NOD/SCID repopulation. **Blood**, 100(8): 2778-2786.

46. Gammaitonia L, Brunoa S, Sanavioa F, Gunettia M, Kollet O, iCavallonia G, Faldac M, Fagiolid F, **Lapidot T**, Aglietta M, & Piacibelloa W. (2003). Ex vivo expansion of human adult stem cells capable of primary and secondary hemopoietic reconstitution. **Exp. Hematology** 31(3): 261-270.

47. Rozovskaia T, Ravid-Amir O, Tillib S, Getz G, Feinstein E, Agrawal H, Nagler A, Rappaport E, Issaeva I, Matsuo Y, Kees U, **Lapidot T**, Lo Coco F, Foa R, Mazo A, Nakamura T, Croce C, Cimino G, Domany E, and Canaani E. (2003). Expression profiles of acute lymphoblastic and myeloblastic leukemias with ALL-1 rearrangements. **PNAS,** 100 (13): 7853-58.

48. Kollet O, Shivtiel S, Chen Y, Suriawinata J, Thung S, Dabeva M, Kahn J, Spiegel A, Dar A, Samira S, Goichberg P, Kalinkovich A, Arenzana-Seisdedos F, Nagler A, Hardan I, Revel M, Shafritz D, & **Lapidot T.** (2003). HGF, SDF-1 and MMP-9 are involved in stress-induced human CD34 + stem cell recruitment to the liver. .**J. Clinical Investigations,** 112:160-169.

49. Peller S, Frenkel J, **Lapidot T,** Kahn J, Rahimi-Levene N, Yona R, Nissim L, Goldfinger N, Sherman D, Rotter V. (2003). The onset of p53-dependent apoptosis plays a role in terminal differentiation of human normoblasts. **Oncogene.** 22(30):4648-55

50. Spiegel A, Kollet O, Peled A, Abel L, Nagler A, Bielorai B, Rechavi G, Vormoor J & **Lapidot T.** (2004). Unique SDF-1 induced activation of human precursor-B ALL cells due to altered CXCR4 expression and signaling. **Blood** , 103(8): 2900-7.

51. Kahn J, Byk T, Jansson L, Petit I, Shivtiel S, Nagler A, Hardan I, Deutsch V, Gazit Z, Gazit D, Karlsson S & **Lapidot T.** (2004). Overexpression of CXCR4 on human $CD34^+$ progenitors increases their proliferation, migration, and NOD/SCID repopulation. **Blood,** 103(8): 2942-9.

52. Avigdor A, Goichberg P, Shivtiel S, Dar A, Peled A, Samira S, Kollet O, Hershovitz R, Alon R, Hradan I, Ben-Hur H, Naor D, Nagler A & **Lapidot T.** (2004). CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34 +stem/progenitor cells to the bone marrow. **Blood** , 103(8):2981-9.

53. Tavor S, Petit I, Porozov S, Avigdor A, Dar A, Leider L, Shem-Tov N, Deutch V, Naparstek E, Nagler A & **Lapidot T.** (2004). CXCR4 regulates migration and development of human AML stem cells in transplanted NOD/SCID mice. **Cancer Research**, 64: 2817-24.

54. Hecht I, Hershkovits R, Shivtiel S, **Lapidot T**, Cohen I, Lider O & Cahalon L. (2004). Heparin-disaccharide affects T cells: inhibition of NF-κB activation,cell migration,and modulation of intracellular signaling. **Journal of Leukocyte Biology,** 75: 1139-46.

55. Bong Kang T, Ben-Moshe T, Varfolomeev E, Pewzner-Jung Y, Yogev N, Jurewicz A, Waisman A, Brenner O, Haffner R, Gustafsson E, Ramakrishnan P, **Lapidot T**, & Wallach D. (2004). Caspase-8 Serves Both Apoptotic and Nonapoptotic Roles. **The Journal of Immunology**, 173:2976–2984.

56. Samira S, Ferrand C, Peled A, Nagler A, Tovbin Y, Ben-Hur H, Taylor N, Globerson A, & **Lapidot T**. (2004) Tumor necrosis factor promotes human T-cell development in nonobese diabetic/severe combined immunodeficient mice. **Stem Cells**, 22(6):1085-100.

57. Petit I, Goichberg P, Spiegel A, Peled A, Brodie C, Seger R, Nagler A, Alon R & **Lapidot T**. (2005). Atypical PKC-ζ regulates SDF-1-mediated migration and development of human CD34+ progenitor cells. **J. Clinical Investigations,** 115:168-176.

58. Zannettino A, Farrugia A, Kortesidis A, Manavis J, To LB, Martin S, Diamond P, Tamamura H, **Lapidot T**, Fujii N, & Gronthos S. (2005). Elevated Serum Levels of SDF-1 are Associated with Increased Osteoclast Activity and Osteolytic Bone Disease in Multiple Myeloma Patients. **Cancer research**, 65: 1700-09.

59. Kortesidis A, Zannettino A, Isenmann S, Shi S, **Lapidot T**, & Gronthos S. (2005). Stromal Derived Factor-1 Promotes the Growth, Survival and Development of Human Bone Marrow Stromal Stem Cells. **Blood** 105: 3793-3801.

60. Byk T, Kahn J, Kollet O, Petit I, Samira S, Shivtiel S, Ben-Hur H, Peled A, Piacibello W, & **Lapidot T**. (2005). Cycling G1 $CD34^{+}/CD38^{+}$ Cells Potentiate the Motility and Engraftment of Quiescent G0 $CD34^{+}/CD38^{-/low}$ SCID Repopulating Cells. **Stem cells**, 23 (4): 561-74.

61. Tavor S, Petit I, Porozov S, Goichberg P, Avigdor A, Sagiv S, Nagler A, Naparstek E, & **Lapidot T**. (2005). Motility, proliferation and egress to the circulation of human AML cells in transplanted Nod/SCID mice, are elastase dependent. **Blood**, In Press.

Invited editorials:

1. Dick J, & **Lapidot T**. (2003). Inside Blood: Stem cells take a short cut to the bone marrow. **Blood** 101 (8): 2901.

2. Kollet O. and **Lapidot T.** (2004). Inside Blood. In-utero transplantation: Hepatic potential of human CD34+/CD45+ cells. **Blood** 104 (8): 2207-8.

Invited reviews:

1. Dick J, **Lapidot T,** & Pflumio P, (1991). Transplantation of normal and leukemic human bone marrow into immune-deficient mice: Development of animal models for human hematopoiesis. **Immunological Reviews** 124: 25-43

2. Dick J, Pflumio F, & **Lapidot T,** (1991). Mouse models for human hematopoiesis. **Seminars in Immunology** 3:367-378

3. Dick J, Sirard C, Pflumio F, & **Lapidot T,** (1992). Murine models of normal and neoplastic human hematopoiesis. **Cancer Surveys** 15, 161-181

4. **Lapidot T**, Pflumio F, & Dick J, (1993). Modeling human hematopoiesis in immunodeficient mice. **Laboratory Animal Science** 43, 2, 147-150

5. Vormoor J, **Lapidot T**, Pflumio F, Risdon G, Patterson B, Broxmeyer H, & Dick J. (1994). SCID mice as an in-vivo model of human cord-blood hematopoiesis. **Blood Cells** 20 (2-3): 316.

6. **Lapidot T**, Fajerman Y. & Kollet O, (1997). Immune deficient SCID and NOD/SCID mice models as functional assays for studying normal and malignant human hematopoiesis. **J. Mol Med** .664-73.

7. **Lapidot T.** (2000). The Roles of the Chemokine SDF-1 and its Receptor CXCR4 in Human Stem Cell Migration and Repopulation of NOD/SCID and B2mnull NOD/SCID mice. In: **Transp. in Hematology and Oncology II,** Springer-Verlag Berlin Heidelberg 2003, P11.

8. **Lapidot T.** (2001). Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice - The role of SDF-1/CXCR4 interactions. **ANN NY ACAD SCI** 938: 83-95

9. **Lapidot T**, & Petit I. (2002). Current understanding of stem cell mobilization: The roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines and stromal cells. **Experimental Hematology**, 30: 973-981.

10. **Lapidot T**, & Kollet O, (2002). The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m^{null} mice. **Leukemia**, 16, 10: 1992-2003.

11. **Lapidot T.** Petit I & Kollet O. (2003) Current understanding of factors influencing stem cell mobilization. In: **Hematology** 2003, p419-37, Education session on Stem Cell Mobilization, 45th Annual Meeting of the American Society of Hematology, San Diego, CA, U.S.A, Dec. 5-9, 2003,

12. **Lapidot T.** and Kollet O. (2004). Mechanism of human CD34+ stem cell homing and mobilization in transplanted immune-deficient NOD/SCID mice. **Macs & more** 8:1,p 14-15.

13. **Lapidot T**, Dar A, & Kollet O. (2005). How do stem cells find their way home? **Blood**, In Press.

Five most cited manuscripts:

14. Lapidot et al. Science 1992.	300 citations.
16. Lapidot et al. Nature 1994.	225 citations.
30. Peled et al. Science 1999.	406 citations.
36. Peled et al. Blood 2000.	157 citations.
44. Petit et al. Nature Immunology 2002.	100 citations.

we should expect great variability in zooxanthellae density. Hence, bleaching events in corals within such lagoons may be frequent and part of the expected cycle of variability.

References and Notes

1. P. W. Glynn, *Coral Reefs* **12**, 1 (1993).
2. L. Muscatine and J. W. Porter, *Bioscience* **27**, 454 (1977).
3. P. W. Glynn, *Trends Ecol. Evol.* **6**, 175 (1995).
4. C. B. Cook, A. Logan, J. Ward, B. Luckhurst, C. J. Berg Jr., *Coral Reefs* **9**, 45 (1990); R. D. Gates, *ibid.* **8**, 193 (1990); T. J. Goreau and R. L. Hayes, *Ambio* **23**, 176 (1994); B. E. Brown, M. D. A. Le Tissier, R. P. Dunne, *Mar. Ecol. Prog. Ser.* **105**, 209 (1994); W. K. Fitt and M. E. Warner, *Biol. Bull.* **189**, 298 (1995).
5. M. P. Lesser, W. R. Stochaj, D. W. Tapley, J. M. Shick, *Coral Reefs* **8**, 225 (1990); D. F. Gleason and G. M. Wellington, *Nature* **365**, 836 (1993).
6. A. Kushmaro, Y. Loya, M. Fine, *Nature* **380**, 396 (1996).
7. O. Hoegh-Gulberg and G. J. Smith, *Mar. Ecol. Prog. Ser.* **57**, 173 (1989).
8. The data were collected from a lagoon near a small coastal village, Trou aux Biches, in the northwest of the island of Mauritius (latitude 20°S). The lagoonal area is about 4.1×10^6 m^2 with an average depth of 2.5 m. There is a high degree of eutrophication in the lagoon with only 20% live coral cover (80 to 90% *Acropora*, and a few occurrences of *Pocillopora* and *Porites*), algal proliferation (50% *Padina*, 10% *Sargassum*, 5% *Turbinaria*, 5% *Valonia*, 5% *Galaxaura*, and 25% other), and a considerable degree of anthropogenic activity (swimming, boating, fishing, snorkeling, water-skiing). A colony was selected that lay in about 2 m of water, depending on the state of the tide. Coral samples were collected by breaking off one live coral tip (2 to 3 cm) from a randomly selected part of the same colony each week.
9. Dissolved oxygen and surface water temperature (at depths of 0.5 to 1.0 m) were taken in situ, and nitrate and phosphate concentrations were determined from water samples in the laboratory. Zooxanthellae were extracted from live coral tips according to Drew's technique (*13*) after decalcifying in 5% HCL. Aliquots of homogeneous extracts were placed on a hemocytometer (improved Neubauer counting chamber, depth 0.1 mm), and zooxanthellae cells were counted under an inverted microscope at ×400. The aluminum foil method of Marsh [J. A. Marsh, *Ecology* **55**, 255 (1970)] was used to calculate the surface area of the coral tip from which the zooxanthellae were extracted. Data on amounts of solar radiation and rainfall in the area were obtained from the Mauritius meteorological office.
10. L. Muscatine, P. G. Falkowski, Z. Dubinsky, P. A. Cook, L. R. McCloskey, *Proc. R. Soc. London Ser. B* **236**, 311 (1989).
11. P. G. Falkowski, Z. Dubinsky, L. Muscatine, L. R. McCloskey, *Bioscience* **43**, 606 (1993).
12. To test for density dependence, we assumed that the change in density was linearly related to the present density. The following model [B. Dennis and M. L. Taper, *Ecol. Monogr.* **64**, 205 (1994)] was fitted:

$$\ln(N_t/N_{t-1}) = a + bN_{t-1} + \sigma Z_t$$

where N_t is the density of zooxanthellae at time t, a and b are constants, σ is a positive constant, and Z_t has a normal distribution with a mean of 0 and a variance of 1, so that σZ_t is the term representing density-independent factors (random shocks). The time between successive points, t and $t + 1$, is 1 week. A density-dependent model has a value for b significantly different from zero. The maximum likelihood estimates for the parameter values are $a = 0.15$, $b = -0.10$, and $\sigma^2 = 0.84$; the probability of the null model being rejected is $P = 0.016$ (as calculated by parametric bootstrapping).
13. E. A. Drew, *J. Exp. Mar. Biol. Ecol.* **9**, 71 (1972).
14. J. Stimson, *ibid.* **214**, 35 (1997).
15. L. R. McCloskey, D. S. Wethey, J. W. Porter, *Monogr. Oceanogr. Methodol.* **55**, 379 (1978).
16. The sea surface temperature shows a seasonal fluctuation, with maximum temperatures in the summer period (December to April) just exceeding 30°C. The minimum temperature was 22.8°C (August 1993) and the maximum was 30.8°C (April 1994). The concentrations of dissolved oxygen (mean = 7.8 ppm, SD = 2.6 ppm), nitrate (mean = 26.9 μg liter^{-1}, SD = 45.7 μg liter^{-1}), and phosphate (mean = 20.0 μg liter^{-1}, SD = 44.1 μg liter^{-1}) all fluctuated greatly over the study period (n = 147).
17. A. M. Szmant, L. M. Ferrer, L. M. Fitzgerald, *Mar. Biol.* **104**, 119 (1990); F. Marubini and P. S. Davies, *ibid.* **127**, 319 (1996).
18. Because dissolved oxygen in the water column is continuous with water in the coelenteron, it may lead to increased oxygen concentration within the coral. High concentrations of oxygen within the coral can precipitate bleaching [M. P. Lesser, *Coral Reefs* **16**, 187 (1997)].
19. Because the data collected were from the tip of the coral and because growth is expected to be greatest at this point [E. H. Gladfelter, *Biol. Bull. (Woods Hole)* **165**, 811 (1983); F. P. Wilkerson, D. Kobayashi, L. Muscatine, *Coral Reefs* **7**, 29 (1988)], the observed variability may be attributable in part to a combination of coral growth and zooxanthellae division to exploit the newly available space [R. J. Jones and D. Yellowlees, *Philos. Trans. R. Soc. London Ser. B* **352**, 457 (1997)].
20. Some of this variability is undoubtedly because different parts of the same colony were sampled and the orientation of the coral branch to incident light is known to affect zooxanthellae density [L. R. McCloskey and L. Muscatine, *Proc. R. Soc. London Ser. B* **222**, 215 (1984); Z. Dubinsky, P. G. Falkowski, J. W. Porter, L. Muscatine, *ibid.*, p. 203]. In addition, it is possible that different strains of zooxanthellae exist in different parts of the colony [R. Rowan, N. Knowlton, A. Baker, J. Jara, *Nature* **388**, 265 (1997)]. Thus, the data collected reflect the normal degree of zooxanthellae variability expected over an entire colony. However, this cannot be the only cause of variation, as the test for density dependence specifically examined and rejected the hypothesis that the sole cause of the variability through time is just random sampling from some distribution of zooxanthellae abundance.

21. We thank S. Davy for reviewing the manuscript and the British Council for sponsoring the link between the University of Mauritius and Imperial College, London and the Link in Marine Environmental Science between the University of Mauritius and the University of Wales, Bangor. The work was also supported by a Natural Environment Research Council (NERC) Advanced Fellowship (H.B.W.) and by a grant from NERC (M.P.H.).

27 May 1998; accepted 23 December 1998

Dependence of Human Stem Cell Engraftment and Repopulation of NOD/SCID Mice on CXCR4

Amnon Peled,[1] Isabelle Petit,[1] Orit Kollet,[1] Michal Magid,[1] Tanya Ponomaryov,[1] Tamara Byk,[1] Arnon Nagler,[3] Herzl Ben-Hur,[4] Ariel Many,[5] Leonard Shultz,[6] Ofer Lider,[1] Ronen Alon,[1] Dov Zipori,[2] Tsvee Lapidot[1]*

Stem cell homing and repopulation are not well understood. The chemokine stromal cell–derived factor-1 (SDF-1) and its receptor CXCR4 were found to be critical for murine bone marrow engraftment by human severe combined immunodeficient (SCID) repopulating stem cells. Treatment of human cells with antibodies to CXCR4 prevented engraftment. In vitro CXCR4-dependent migration to SDF-1 of CD34$^+$CD38$^{-/low}$ cells correlated with in vivo engraftment and stem cell function. Stem cell factor and interleukin-6 induced CXCR4 expression on CD34$^+$ cells, which potentiated migration to SDF-1 and engraftment in primary and secondary transplanted mice. Thus, up-regulation of CXCR4 expression may be useful for improving engraftment of repopulating stem cells in clinical transplantation.

Stem cells within the bone marrow microenvironment actively maintain continuous production of all mature blood cell lineages throughout life. These rare primitive cells are functionally defined by their ability to home to the bone marrow and to durably repopulate transplanted recipients with both myeloid and lymphoid cells (*1*, *2*). Several groups have established in vivo models for engrafting human stem cells (*3*–*8*). We developed a functional in vivo assay for primitive human SCID repopulating cells (SRCs) based on their ability to repopulate the bone marrow of intravenously transplanted SCID or nonobese diabetic SCID (NOD/SCID) mice with high levels of both myeloid and lymphoid cells (*5*, *6*, *8*).

Chemokines are cytokines that are best known for their ability to selectively attract subsets of leukocytes to sites of inflammation (*9*). The role that chemokines and their receptors play in homing and repopulation of human stem cells is not fully understood. The

[1]Department of Immunology and [2]Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel. [3]Hadassah University Hospital Jerusalem, 91120, Israel. [4]Kaplan Medical Center, Rehovot 76100, Israel. [5]Sourasky Medical Center, Tel Aviv 64239, Israel. [6]The Jackson Laboratory, Bar Harbor, ME 04609, USA.

*To whom correspondence should be addressed. E-mail: litsvee@weizmann.weizmann.ac.il

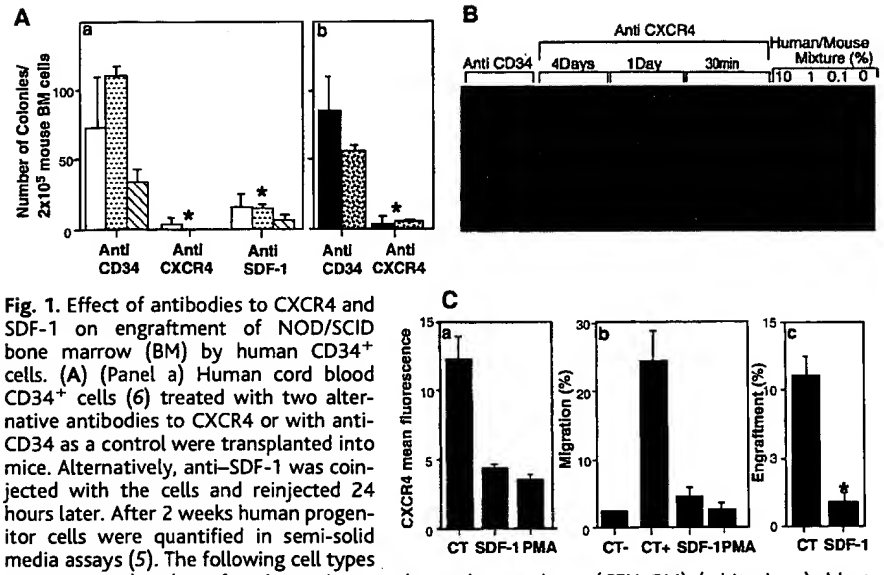


Fig. 1. Effect of antibodies to CXCR4 and SDF-1 on engraftment of NOD/SCID bone marrow (BM) by human $CD34^+$ cells. **(A)** (Panel a) Human cord blood $CD34^+$ cells (6) treated with two alternative antibodies to CXCR4 or with anti-CD34 as a control were transplanted into mice. Alternatively, anti–SDF-1 was coinjected with the cells and reinjected 24 hours later. After 2 weeks human progenitor cells were quantified in semi-solid media assays (5). The following cell types were counted: colony-forming unit–granulocyte/macrophage (CFU-GM) (white bars), blast-forming unit–erythroid (BFU-E) (dashed bars), and multilineage colony (CFU-GEMM) (striped bars). Data are average ± SE ($^*P < 0.01$, as determined by paired Student's *t* test) of three experiments. (Panel b) Human bone marrow (black bars) or mobilized peripheral blood (stippled bars) $CD34^+$ cells were treated with the indicated antibodies and transplanted into NOD/SCID mice, and total human progenitors were quantified after 1 month as for panel a. **(B)** Antibodies to CXCR4 were injected at the indicated times after transplantation of NOD/SCID mice with cord blood $CD34^+$ cells. Control cells were incubated with anti-CD34. After 2 weeks, bone marrow was assayed by Southern blot for human DNA with a human-specific α satellite probe (5). **(C)** Cord blood $CD34^+$ cells were either not treated (CT) or treated for 24 hours with SDF-1 or PMA. (Panel a) CXCR4 surface expression of $CD34^+$ cells. (Panel b) Transwell migration assay (13) of untreated cells without SDF-1 (CT−) or with SDF-1 (CT+), and migration to SDF-1 of treated cells. (Panel c) The percent of human cells in NOD/SCID mice 1 month after transplantation was determined by FACS analysis with antibodies to human CD45. Data are average ± SE ($^*P < 0.01$) of three experiments.

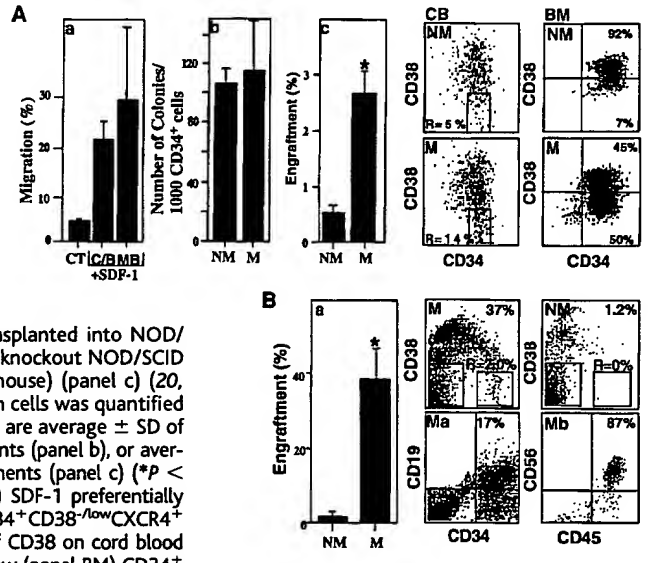


Fig. 2. SDF-1 induces the migration of SRCs. **(A)** (Panel a) Transwell migration assay with $CD34^+$ cord blood (C), bone marrow (B), or mobilized peripheral blood (MB) cells (13, 14). CT, migration without SDF-1. SDF-1 migrating (M) and nonmigrating (NM) cells were assayed for progenitors (panel b) or transplanted into NOD/SCID or β_2-microglobulin knockout NOD/SCID mice (3×10^4cells per mouse) (panel c) (20, 23). The percent of human cells was quantified as in Fig. 1C, panel c. Data are average ± SD of 11 (panel a) or 3 experiments (panel b), or average ± SE of three experiments (panel c) ($^*P <$ 0.01). (Panels CB and BM) SDF-1 preferentially induces migration of $CD34^+CD38^{-/low}CXCR4^+$ cells. Surface expression of CD38 on cord blood (panel CB) and bone marrow (panel BM) $CD34^+$ cells was analyzed by flow cytometry on SDF-1 migrating (M) or nonmigrating (NM) cells. R gates $CD34^+/CD38^-$ cells. **(B)** Sorted cord blood $CD34^+CD38^{-/low}$ cells, (Panel a) SDF-1 migrating (M) or nonmigrating (NM) cells were transplanted into NOD/SCID mice (3×10^4cells per mouse). After 6 weeks, percent of engraftment was quantified as in Fig. 1C, panel c. Data are average ± SE ($^*P < 0.01$) of three experiments. Phenotype analysis of engrafted M and NM cells. Numbers indicate percent of human cells. (Panels Ma and Mb) The presence of human lymphoid $CD45^+CD19^+$ pre-B cells (panel Ma) and progenitors for human $CD45^+CD56^+$ natural killer cells (panel Mb) is shown.

chemokine SDF-1 (10) binds to its receptor CXCR4, which is expressed on many cell types, including some $CD34^+CD38^-$ cells (11, 12). In vitro SDF-1 attracts certain $CD34^+CXCR4^+$ cells, and in vivo it is produced by bone marrow stromal cells as well as by epithelial cells in many organs (11, 13, 14). Mice that lack SDF-1 or do not express CXCR4 exhibit many defects, including the absence of both lymphoid and myeloid hematopoiesis in the fetal bone marrow (10, 15). Overexpression of human CD4 and CXCR4 receptors on murine $CD4^+$ T cells led to enhanced homing of these cells to the murine bone marrow (16).

To examine the in vivo role of SDF-1 and its receptor CXCR4 in migration and repopulation by human SRCs, we treated $CD34^+$-enriched cord blood cells either with two different antibodies to CXCR4 or with control antibodies to CD34 (anti-CD34) before transplantation of NOD/SCID mice (Fig. 1A, panel a). Only anti-CXCR4 reduced engraftment. Similar treatment of human $CD34^+$-enriched cells from mobilized peripheral blood or adult bone marrow also resulted in inhibition of engraftment (Fig. 1A, panel b). Antibodies to SDF-1 coinjected with human $CD34^+$ cord blood cells and readministered after 24 hours significantly reduced the level of engraftment (Fig. 1A, panel a). The first 24 hours were critical to the engraftment process. Antibodies administered intraperitoneally 30 min after transplantation blocked engraftment (Fig. 1B). Antibodies administered 24 hours later reduced engraftment less effectively and when administered 4 days after transplantation were completely ineffective (Fig. 1B) (17).

The effects of SDF-1 desensitization and CXCR4 down-regulation on the ability of human $CD34^+$ cells to migrate and engraft NOD/SCID mice were further studied. SDF-1 and phorbol esters [phorbol 12-myristate 13-acetate (PMA)] cause internalization and down-regulation of CXCR4 surface expression on human $CD4^+$ T cells (18). Cord blood $CD34^+$ cells were incubated overnight with high doses of SDF-1. Cells were subsequently washed and tested for CXCR4 expression and migration to SDF-1 in a transwell assay. Treatment of $CD34^+$ cells with SDF-1 or PMA reduced CXCR4 cell surface expression (Fig. 1C, panel a) and abolished the migration of $CD34^+$ cells in response to SDF-1 (Fig. 1C, panel b) (19). Prolonged treatment of $CD34^+$ cells with SDF-1 significantly blocked the engraftment of transplanted NOD/SCID mice (Fig. 1C, panel c). Thus, SDF-1 probably affects SRC engraftment by mediating chemotaxis to the bone marrow, linking migration to SDF-1 in vitro to human stem cell function in vivo.

The migration potential of human $CD34^+$ cells from cord blood, bone marrow, or mo-

bilized peripheral blood was tested in vitro in a transwell assay. Consistent with previous studies (*13*), 20 to 25% of cord blood and bone marrow $CD34^+$ cells migrated in response to a chemotactic gradient of SDF-1 in all donors tested (Fig. 2A, panel a). Migration of mobilized peripheral blood $CD34^+$ cells from multiple donors in response to SDF-1 was variable (between 8 to 60%), suggesting the involvement of SDF-1 in the mobilization process (Fig. 2A, panel a). The migrating and nonmigrating $CD34^+$ cell populations did not differ in the incidence of progenitor cells, as determined by in vitro colony assays (Fig. 2A, panel b); however, the engraftment potential of the migrating and nonmigrating $CD34^+$ cells was different. Equal numbers of migrating (M) and nonmigrating (NM) $CD34^+$ cells were washed and transplanted into NOD/SCID or NOD/SCID β_2 microglobulin knockout mice (*20*). Whereas mice transplanted with nonmigrating cells were poorly engrafted, mice transplanted with migrating cells were significantly better engrafted (Fig. 2A, panel c). The low concentrations of SDF-1 and the limited exposure time caused only a transient decrease of CXCR4 expression that did not prevent engraftment. These results are further evidence for the link between in vitro motility to SDF-1 and in vivo stem cell function.

Although only 20 to 25% of cord blood $CD34^+$ cells migrated toward SDF-1, this population contained a significantly higher percentage of primitive $CD34^+CD38^-$ cells than did nonmigrating cells (Fig. 2A, panel CB). In $CD34^+$ cells from bone marrow, the proportion of immature $CD34^+CD38^{-/low}$ cells migrating to SDF-1 was larger than in cord blood (Fig. 2A, panel BM). Nevertheless, most cord blood $CD34^+CD38^-$ cells (60%) did not migrate to SDF-1, demonstrating that $CD34^+CD38^-$ cells are a heterogeneous population composed mostly of nonmigrating cells. Sorted $CD34^+CD38^{-/low}$ cord blood cells from different donors were evaluated for their ability to migrate toward a chemotactic gradient of SDF-1 in vitro on the basis of surface CXCR4 expression and for their content of SRCs in vivo. Only 26% (±7%) of the $CD34^+CD38^{-/low}$ cells migrated to a gradient of SDF-1 in the transwell assay. Transplantation of migrating $CXCR4^+$ cells into NOD/SCID mice resulted in high levels of multilineage engraftment (Fig. 2B). This was reflected in the engraftment of primitive $CD34^+CD38^-$ cells (Fig. 2B, panel M) and lymphoid (Fig. 2B, panels Ma and Mb), and myeloid colony-forming cells. In contrast, little engraftment was observed with nonmigrating $CXCR4^{-/low}$ cells (Fig. 2B, panel NM). Thus, the $CD34^+CD38^{-/low}CXCR4^+$ migrating cell population representing less than one-third of all $CD34^+CD38^{-/low}$ cells engrafts the murine bone marrow with SRCs.

Kim and Broxmeyer have demonstrated that stem cell factor (SCF) attracts $CD34^+$ cells, increases their motility, and synergizes with SDF-1, increasing migration to both cytokines in vitro (*21*). Unexpectedly, prolonged (24- to 48-hour) stimulation of mobilized peripheral blood $CD34^+$ cells with SCF resulted in increased CXCR4 expression (Fig. 3A), enhanced migration toward SDF-1 (Fig. 3B), and enhanced engraftment potential dependent on the exposure time to SCF (Fig. 3C). Engraftment potential was similarly increased when only half the cell number was injected after 40 hours of SCF treatment, compared with 16 hours of exposure or untreated cells transplanted at time 0 (Fig. 3D). Thus, enhanced CXCR4-dependent migration to SDF-1 was accompanied by an increase in the SRC fraction. Incubation of SCF-stimulated, mobilized peripheral blood $CD34^+$ cells with anti-CXCR4 prevented engraftment (Fig. 3C). Sorted $CD34^+CD38^{-/low}CXCR4^{-/low}$ cord blood cells that did not migrate toward SDF-1 were either transplanted or treated with SCF for 48 hours. Whereas nontreated cells had low engraftment efficiency (Fig. 4A), SCF treatment resulted in increased migration

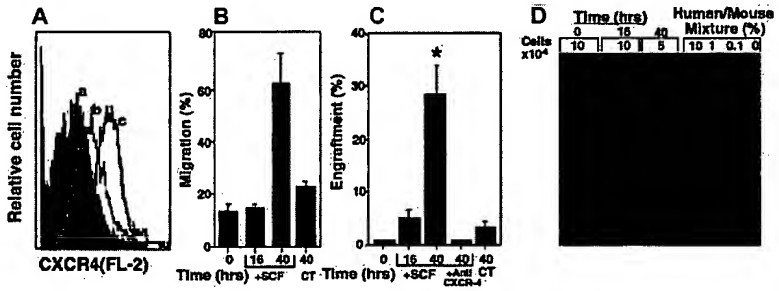


Fig. 3. SCF potentiates CXCR4 expression, cell migration, and SRC engraftment. (**A**) Mobilized peripheral blood $CD34^+$ cells stained with control antibody (curve a) or with anti-CXCR4 before (curve b) or after (curve c) 40 hours of treatment with SCF. (**B**) SDF-1 transwell migration of untreated (0), SCF-treated (16 and 40 hours), or control cells cultured for 40 hours without SCF (CT). Data are average ± SE of three experiments. (**C**) Percent of engraftment in NOD/SCID mice transplanted with 2×10^5 cells before (0) or after 16 or 40 hours of exposure to SCF and 40 hours of exposure to SCF followed by incubation with anti-CXCR4 (+ anti CXCR4). Control cells (CT) as in (B). Percent of engraftment was quantified as in Fig. 1C, panel c. Data are average ± SE (*$P <$ 0.01, SCF 40 hours versus 0 hours, SCF+anti-CXCR4, and CT 40 hours) of three mice per treatment, in a representative experiment. (**D**) Exposure times of mobilized peripheral blood $CD34^+$ to SCF as in (C). At time 0 and after 16 hours 1×10^5 cells per mouse were transplanted, and after 40 hours 0.5×10^5 cells per mouse were transplanted. Human engraftment was quantified after 1 month by Southern blot analysis.

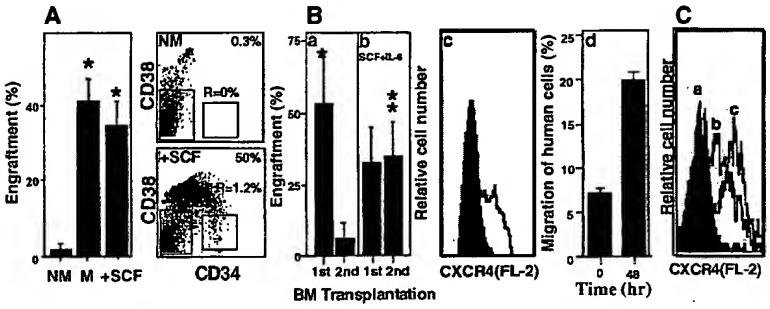


Fig. 4. Increase in SRCs and of stem cell self-renewal by up-regulation of CXCR4 expression. (**A**) Sorted $CD34^+CD38^{-/low}$ cord blood cells migrating toward SDF-1 were transplanted into NOD/SCID mice (3×10^4 cells per mouse) (M). Nonmigrating cells were either injected directly (NM) or treated with SCF for 48 hours and then injected (+SCF). After 6 weeks engraftment levels were quantified as in Fig. 1C, panel c. Data in the left panel are average ± SE (*$P < 0.01$) of four experiments. (**B**) Bone marrow cells from mice transplanted 4 to 6 weeks before with human cord blood $CD34^+$ cells in panels a and b were retransplanted untreated (2nd in panel a) or after SCF and IL-6 treatment for 48 hours (panel b) into secondary β_2-microglobulin knockout NOD/SCID mice. Data in panels a and b are the average ± SE of four experiments (panel a, *$P < 0.01$, 1st versus 2nd; **$P < 0.05$, 2nd in panel b versus 2nd in panel a). (Panel c) Human CXCR4 expression on cord blood cells from transplanted mice immediately labeled (solid) or after 48 hours treatment with SCF and IL-6 (open). (Panel d) SDF-1 migration of cord blood cells from the marrow of transplanted mice before and after treatment with SCF and IL-6 for 48 hours. Data in panel d are the average of triplicates in a representative experiment. (**C**) Cord blood $CD34^+$ cells were stained with control antibody (curve a) or antibody to CXCR4 after a 48-hour exposure to SCF (curve b) or SCF and IL-6 (curve c). Percent of engraftment in (A) and (B) was quantified as in Fig. 1C, panel c.

toward SDF-1 and efficient engraftment by converted $CD34^+CD38^{-/low}CXCR4^+$ cells, properties that were similar to those of the original migrating fraction (M) (Fig. 4A).

Self-renewal of stem cells can only be determined by their ability to also repopulate secondary transplanted recipients with high numbers of both myeloid and lymphoid cells. Consistent with previous studies, secondary transplanted mice that received untreated human cells showed little engraftment (Fig. 4B, panel a) (*22*). Human interleukin-6 (IL-6) synergizing with SCF induced high levels of CXCR4 expression on $CD34^+$ cord blood cells (Fig. 4C). Incubation of bone marrow cells from primary transplanted mice with SCF and IL-6 for 48 hours resulted in up-regulation of surface CXCR4 expression (Fig. 4B, panel c) and increased migration of human progenitor cells to SDF-1 in vitro (Fig. 4B, panel d). Transplantation of similar numbers of human cells from the bone marrow of primary transplanted mice after treatment with these cytokines resulted in higher engraftment levels in secondary transplanted mice compared with mice transplanted with untreated cells (Fig. 4B, panel b versus panel a). Thus, by up-regulating surface CXCR4 expression on primitive cells, the population of self-renewing $CD34^+CD38^{-/low}$ SRC stem cells could be increased.

Our data provide evidence that CXCR4-dependent migration to SDF-1 is essential for human stem cell function in NOD/SCID mice. We characterized SRCs further as $CD34^+CD38^{-/low}CXCR4^+$ stem cells and showed that $CD34^+CD38^{-/low}CXCR4^{-/low}$ cells can be converted into functional $CXCR4^+$ stem cells by cytokine treatment. This suggests that migration to SDF-1 is associated with localization of stem cells in the bone marrow, permitting differentiating cells with reduced migration levels to exit into the blood circulation. In conclusion, our findings define human $CD38^{-/low}CXCR4^+$ cells as stem cells endowed with migration and repopulation potential and provide insights into human stem cell biology.

References and Notes

1. S. Morrison, N. Uchida, I. Weissman, *Annu. Rev. Cell Dev. Biol.* **11**, 35 (1995).
2. H. J. Sutherland *et al.*, *J. Hematother.* **4**, 3 (1995).
3. J. M. McCune *et al.*, *Science* **241**, 1632 (1988).
4. J. A. Nolta, M. B. Hanley, D. B. Kohn, *Blood* **83**, 3041 (1994).
5. T. Lapidot *et al.*, *Science* **255**, 1137 (1992).
6. A. Larochelle *et al.*, *Nature Med.* **2**, 1329 (1996).
7. C. I. Civin, G. A. Porada, M. J. Lee, L. Terstappen, E. D. Zanjani, *Blood* **88**, 4102 (1996).
8. J. D. Cashman *et al.*, *ibid.* **89**, 4307 (1997).
9. B. A. Premack and T. J. Schall, *Nature Med.* **2**, 1174 (1996).
10. T. Nagasawa *et al.*, *Nature* **382**, 635 (1996).
11. C. C. Bleul, R. C. Fuhlbrigge, J. M. Casasnovas, A. Aiuti, T. A. Springer, *J. Exp. Med.* **184**, 1101 (1996).
12. M. Deichmann, R. Kronenwett, R. Haas, *Blood* **89**, 3522 (1997).
13. A. Aiuti, I. J. Webb, C. Bleul, T. Springer, J. C. Gutierrez-Ramos, *J. Exp. Med.* **185**, 111 (1997).
14. M. Shirozu *et al.*, *Genomics* **28**, 495 (1995).
15. Y. Zou, A. H. Kottmann, M. Kuroda, I. Taniuchi, D. R. Littman, *Nature* **393**, 595 (1998).
16. S. Sawada *et al.*, *J. Exp. Med.* **187**, 1439 (1998).
17. Treatment of $CD34^+$ cells with either of the two antibodies to CXCR4 did not cytotoxically damage the cells, as judged by their normal formation of colonies in semisolid media.
18. N. Signoret *et al.*, *J. Cell Biol.* **139**, 651 (1997).
19. Colony formation in vitro was not affected.
20. S. W. Christiansen *et al.*, *J. Immunol.* **158**, 3578 (1997).
21. C. H. Kim and H. E. Broxmeyer, *Blood* **91**, 100 (1998).
22. G. Spangrude, D. Brooks, D. Tumas, *ibid.* **85**, 1006 (1995).
23. Human cells were obtained after informed consent according to procedures approved by the Weizmann Committee. In all experiments, samples of the same initial cell pool were compared. Differences in the results are due to the different $CD34^+$ cell sources (cord blood, bone marrow, and mobilized peripheral blood). $CD34^+$ enrichment, flow cytometry, and fluorescence-activated cell sorting (FACS) were performed as previously described (*5*, *6*). SDF-1 (125 ng/ml, R&D Systems) transmigration assays were done as previously described (*13*) with 2×10^5 $CD34^+$ cells. Percents in the results represent percent of initial 2×10^5 cells in the migrating and nonmigrating cell fractions. The sources for the reagents are as follows: PMA (100 ng/ml), was purchased from Sigma, stem cell factor (SCF) and IL-6 (50 ng/ml) from R&D Systems, and antibodies to CXCR4 from Pharmingen [12g5 monoclonal antibody [immunoglobulin G2a (IgG2a)]] or R&D Systems [MBA171 monoclonal antibody (IgG2a)] (10 μg per 2×10^5 cells). CXCR4 expression was always analyzed by double staining with anti-CD34. Polyclonal anti–SDF-1 (10 μg per mouse, R&D Systems) was injected intravenously with the cells (2×10^5 cells per mouse) and 24 hours later injected again intraperitoneally. Control cells were incubated with anti-CD34 [IgG1, Becton Dickinson, 10 μg per 2×10^5 cells]. Human lymphoid and myeloid cells were immunostained with anti-CD45 (Immuno Quality Products, Groningen, Netherlands), anti-CD19, and anti-CD56 (Coulter). Natural killer cells differentiated into mature $CD56^+$ cells after incubation with human SCF (100 ng/ml) and human IL-15 (100 ng/ml, R&D Systems) for 10 days. NOD/SCID, and NOD/SCID β_2-microglobulin knockout (*20*) mice were bred and maintained under defined flora in intraventilated cages and transplanted by injection into the tail vein after sublethal (375R) irradiation according to established protocols (*5*, *6*) approved by the Weizmann animal ethics committee. Southern (DNA) blot analysis with a human-specific α satellite probe and human-specific progenitor assays were done as previously described (*5*, *6*). Percent engraftment always indicates the percent of either human DNA or of human CD45 cells in the mouse bone marrow. The levels of engraftment were dependent on the injected cell dose, the duration of the experiment, and the source of human $CD34^+$ cells. Cells were cultured either in serum-free media as previously described (*6*) or in media supplemented with 10% fetal calf serum.

24. Supported by grants from the Israel Academy of Science and the Israel Cancer Research Fund (T.L), the Germany MINERVA grant (A.P.), a grant from the Balfour Peisner Bone Marrow Cancer Research Fund (I.P), a grant from the Israel Ministry of Science (O.K.), and NIH grant A130389 (L.S.). We thank Y. Yarden, E. Canaani, M. Revel, P. Lonai, M. Feldman, and A. B. David for critically reviewing this manuscript and J. Dick for NOD/SCID mice, his pluripotent help and support, and for critically reviewing the revised version of the manuscript.

23 October 1998; accepted 28 December 1998

CD3- and CD28-Dependent Induction of PDE7 Required for T Cell Activation

Linsong Li,[1] Cassian Yee,[2] Joseph A. Beavo[1]*

Costimulation of both the CD3 and CD28 receptors is essential for T cell activation. Induction of adenosine 3′,5′-monophosphate (cAMP)–specific phosphodiesterase-7 (PDE7) was found to be a consequence of such costimulation. Increased PDE7 in T cells correlated with decreased cAMP, increased interleukin-2 expression, and increased proliferation. Selectively reducing PDE7 expression with a PDE7 antisense oligonucleotide inhibited T cell proliferation; inhibition was reversed by blocking the cAMP signaling pathways that operate through cAMP-dependent protein kinase (PKA). Thus, PDE7 induction and consequent suppression of PKA activity is required for T cell activation, and inhibition of PDE7 could be an approach to treating T cell–dependent disorders.

Activation of peripheral T cells in vivo by an antigen-presenting cell is a result of the engagement of both the T cell receptor–CD3 complex (TCR-CD3) and the CD28 costimulatory receptor. When both receptors are occupied by their appropriate ligands, T cells are stimulated to proliferate and produce interleukin-2 (IL-2), whereas occupation of the T cell receptor alone favors T cell anergy or apoptosis (*1*). Occupation of the CD28 receptor alone appears to have no obvious effect on T cells; nevertheless, CD28 costimulation is required for full activation of CD4 T helper cells, if not all T cells (*2*). Why is CD28 costimulation required for T cell activation? One possible reason has been suggested by

[1]Department of Pharmacology and Molecular and Cellular Biology Program, Box 357280, University of Washington School of Medicine, Seattle, WA 98195, USA. [2]Clinical Research Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA 98109, USA.

*To whom correspondence should be addressed. E-mail: Beavo@u.washington.edu

Regulation of the Chemokine Receptor CXCR4 by Hypoxia

Tiziana Schioppa,[1] Badarch Uranchimeg,[5] Alessandra Saccani,[1] Subhra K. Biswas,[1] Andrea Doni,[1] Annamaria Rapisarda,[5] Sergio Bernasconi,[1] Simona Saccani,[3] Manuela Nebuloni,[4] Luca Vago,[4] Alberto Mantovani,[1,2] Giovanni Melillo,[5] and Antonio Sica[1]

[1]*Istituto di Ricerche Farmacologiche "Mario Negri," 20157 Milan, Italy*
[2]*Centro di Eccellenza per l'Innovazione Diangnostica e Terapeutica, Institute of Pathology, State University of Milan, 20133 Milan, Italy*
[3]*Institute for Research in Biomedicine, Bellinzona, CH6500 Switzerland*
[4]*Institute of Pathology, Department of Clinical Sciences "L. Sacco," University of Milan, 20157 Milan, Italy*
[5]*Development Therapeutic Program, Tumor Hypoxia Laboratory, Science Applications International Corporation-Frederick, Inc., National Cancer Institute, Frederick, MD 21702*

Abstract

Cell adaptation to hypoxia (Hyp) requires activation of transcriptional programs that coordinate expression of genes involved in oxygen delivery (via angiogenesis) and metabolic adaptation (via glycolysis). Here, we describe that oxygen availability is a determinant parameter in the setting of chemotactic responsiveness to stromal-derived factor 1 (CXCL12). Low oxygen concentration induces high expression of the CXCL12 receptor, CXC receptor 4 (CXCR4), in different cell types (monocytes, monocyte-derived macrophages, tumor-associated macrophages, endothelial cells, and cancer cells), which is paralleled by increased chemotactic responsiveness to its specific ligand. CXCR4 induction by Hyp is dependent on both activation of the Hyp-inducible factor 1 α and transcript stabilization. In a relay multistep navigation process, the Hyp–Hyp-inducible factor 1 α–CXCR4 pathway may regulate trafficking in and out of hypoxic tissue microenvironments.

Key words: cell migration • SDF-1/CXCL12 receptor (CXCR4) • low oxygen concentration • hypoxia-inducible factor 1 (HIF-1)

Introduction

Oxygen homeostasis represents an important organizing principle for human development and physiology (1). The essential requirement for oxidative phosphorylation to generate ATP is balanced by the risk of oxidative damage to cellular lipids, nucleic acids, and proteins. As a result, cellular and systemic O_2 concentrations are tightly regulated via short- and long-acting response pathways that affect the activity and expression of a multitude of cellular proteins (2). Dysregulation of O_2 homeostasis is found in inflammatory and cardiovascular diseases, cancer, cerebrovascular disease, and chronic obstructive pulmonary disease. Recent papers have begun to delineate the molecular basis of cellular and systemic mechanisms of O_2 homeostasis and the most global regulator identified to date is the transcriptional activator hypoxia (Hyp)-inducible factor 1 (HIF-1) composed of the HIF-1α and HIF-1β subunits (1, 3).

During migration and invasion of normal and pathological tissues, cells may encounter different oxygen levels, due to poor or altered vascularization, and recent evidence has suggested that chemotaxis is a cell function which may be affected by oxygen availability (4, 5). Leukocyte trafficking, an event which plays a central role in fundamental functions of multicellular organisms, including tissue remodelling,

Address correspondence to Antonio Sica, Istituto di Ricerche Farmacologiche "Mario Negri," via Eritrea 62, 20157 Milan, Italy. Phone: 39-02-2390-14530; Fax: 39-02-2332-00231; email: sica@marionegri.it; or Giovanni Melillo, Development Therapeutic Program, Tumor Hypoxia Laboratory, Science Applications International Corporation-Frederick, Inc., National Cancer Institute, Frederick, MD 21701. Phone: (301) 846-5050; Fax: (301) 846-6081; email: melillo@dtpax2.ncifcrf.gov

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; DFX, desferrioxamine; HIF-1, Hyp-inducible factor 1; HRE, Hyp responsive element; HUVEC, human umbilical vein endothelial cell; Hyp, hypoxia; MDM, monocyte-derived macrophage; MEF, mouse embryonic fibroblast; Norm, normoxia; pVHL, von Hippel–Lindau tumor suppressor protein; SDF-1, stromal-derived factor 1; TAM, tumor-associated macrophage; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau tumor suppressor protein.

defense, and pathology, is orchestrated by a superfamily of small proteins termed chemokines, which are essential players in immune and inflammatory reactions as well as in infections (6–8). Based on a cysteine motif, chemokines have been classified into a CXC, CC, C, and CX3C family, and >37 members are identified in humans to date. Chemokines interact with 7 transmembrane domain G-protein–coupled receptors and, so far, 10 CC (CCR1-10), 6 CXC (CXCR1-6), 1 CX3C (CXCR1), and 1 C (XCR1) receptors have been identified (9). Emerging evidence shows that the expression and function of G-protein–coupled receptors is strictly controlled by cytokines and other microenvironmental signals, such as Hyp (10–12), and that the regulation of receptor expression during cell activation and deactivation is as important as the regulation of chemokine production for tuning the chemokine system (13).

Experimental and clinical studies point to the fundamental pathophysiological role of Hyp in inflammatory (14, 15) and neoplastic diseases (16). In particular, tumor Hyp is a consequence of a structurally and functionally disturbed microcirculation and, in some cases, of a reduced O_2-carrying capacity of the blood due to tumor-associated anemia. Hyp in human tumors by itself has been shown to contribute to resistance to standard anticancer therapies, and recent data suggest that O_2-deprived tumor cells are predisposed to a more malignant phenotype (i.e., tumor cells are likely to be more metastatic and/or invasive; reference 16). Solid tumors consist of malignant cells and stroma, which includes new blood vessels, matrix components and cells responsible for their production, a fibrin-gel matrix, and inflammatory leukocytes (17, 18). Thus, tumor stromal cells encounter low oxygen conditions and, in particular, tumor-associated macrophages (TAMs) have been reported to localize preferentially in the hypoxic areas of tumors (4, 5, 19, 20). Based on this evidence, we investigated the effect of Hyp on the expression and function of chemokine receptors. We observed that Hyp selectively augments CXCR4 expression through HIF-1 activation in human monocytes, macrophages, endothelial cells, and cancer cells. The identification of the Hyp–HIF-1α–CXCR4 pathway provides novel insights into the mechanisms controlling cell migration in hypoxic regions, with potential relevance in the pathogenesis of human diseases. The Hyp–HIF-1α–CXCR4 pathway is likely to regulate the migration and localization of diverse cell types in tissues.

Materials and Methods

Cells and Culture Conditions. Human monocytes were separated from peripheral blood of human healthy donors by Percoll gradient centrifugation (10). Monocytes (>98% pure as assessed by morphology) were resuspended at 10^7/ml in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and antibiotics. All reagents contained <0.125 EU/ml of endotoxin as checked by limulus amebocyte lysate assay (Microbiological Associates). Monocyte-derived macrophages (MDMs) were derived from freshly isolated monocytes (3–5 × 10^6 cells/ml) after incubation for 5 d in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, antibiotics, and 40% autologous serum on hydrophobic petriperm plates (Sigma-Aldrich) as described previously (21).

TAMs were obtained from ascitic fluids collected from untreated patients with histologically confirmed epithelial ovarian carcinoma admitted to the Department of Obstetric and Gynecology, S. Gerardo Hospital, Monza, Italy. All patients had cancer classified as stage II, III, or IV. Ascitic fluid was collected and centrifuged. Cells pellet was resuspended in RPMI 1640 medium without serum and layered on top of a Ficoll-Hypaque cushion to prepare mononuclear cells. Purification of peritoneal macrophages was further conducted by two subsequent adherence steps for 45 min each in RPMI 1640 medium without serum. After adherence procedures, cells were repeatedly washed with saline to remove all nonadherent cells. The adherent cells were rested with complete medium overnight at 37°C and subsequently stimulated as indicated in the test.

Human endothelial cells were obtained from umbilical veins and cultured as described previously (22). We used routinely confluent cells at second to sixth passages. Cells at the concentration of 1.5 × 10^4/0.2 ml were cultured for 24 h in flat-bottomed 96-well plates (Falcon) in M199 medium with 20% FCS, supplemented with 50 μg/ml of endothelial cell growth supplement (Collaborative Research Inc.) and 100 μg/ml heparin. Cells were maintained at 37°C in a humidified incubator containing 20% O_2, 5% CO_2, and 75% N_2. For hypoxic conditions, cells were cultured in an atmosphere-controlled culture chamber (Bellco Glass) containing a gas mixture composed of 94% N_2, 5% CO_2, and 1% O_2.

Mouse embryonic fibroblasts (MEFs), wild type, and HIF-$1\alpha^{-/-}$ were a gift from G. Semenza (The Johns Hopkins University School of Medicine, Baltimore, MD) and were routinely maintained in DMEM (Invitrogen and Life Technologies) supplemented with 10% heat-inactivated FBS (Whittaker Bioproducts), 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine (all purchased from Invitrogen and Life Technologies). MCF-7 (human breast cancer cells), CAOV3 (human ovarian cancer cell line), 786.0 (36), and WT2 human renal cancer cells (23) were routinely maintained in RPMI 1640 medium (Whittaker Bioproducts) supplemented with 5% heat-inactivated FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Cells were maintained at 37°C in a humidified incubator containing 20% O_2, 5% CO_2 in air (referred to as normoxic conditions). Hyp treatment was performed by placing cells in a modular incubator chamber (Billupus-Rothemberg Inc.) and flushing with a mixture of 1% O_2, 5% CO_2, and 94% nitrogen for 20 min. The chamber was placed at 37°C.

FACS® Analysis. Cell staining was performed using mouse monoclonal anti–human CXCR4 antibody (clone 12G5; BD Biosciences) and an irrelevant isotype-specific control mouse, IgG2a, κ (UPC10; Sigma-Aldrich) followed by FITC-conjugated, isotype-matched affinity-purified, goat anti–mouse antibody (Southern Biotechnology Associates, Inc.).

Cytokines and Reagents. Human recombinant CXCL12/stromal-derived factor 1 (SDF-1) and CCL5/regulated on activation normal T cell expressed and secreted were from PeproTech. Desferrioxamine (DFX) and actinomycin D (used at 1 mg/ml) were purchased from Sigma-Aldrich.

Migration Assay. Monocyte migration was evaluated using a chemotaxis microchamber technique as described previously (10). 27 ml of chemoattractant solution or control medium (RPMI 1640 with 1% FCS) was added to the lower wells of a chemotaxis

chamber (Neuroprobe). A polycarbonate filter (5 μm pore size; Neuroprobe) was layered onto the wells and covered with a silicon gasket and the top plate. 50 ml of cell suspension (1.5×10^6/ml fresh human monocytes) was preincubated for 16 h in the presence of 400 μM DFX and seeded in the upper chamber. The chamber was incubated at 37°C in air with 5% CO_2 for 90 min. At the end of the incubation, filters were removed and stained with Diff-Quik (Baxter), and 10 high-power oil immersion fields were counted. Cancer cell migration was assayed as described previously (24) by using Falcon transwells (24-well format, 8-μM pore; BD Biosciences). 0.5 ml of media containing 5×10^5 cells was added to the upper chamber, and 0.5 ml of medium alone or media supplemented with CXCL12 was added to the lower chamber. After overnight incubation in hypoxic conditions, cells on the upper surface of the filter were removed using a cotton wool swab. Migrated cells on the lower surface were stained using DiffQuick (Dade Behring). For each transwell, the number of migrated cells in 10 medium-power fields (20×) was counted. For human umbilical vein endothelial cell (HUVEC) migration (25), polycarbonate filters (5 μm pore size, polyvinylpyrrolidine free) were soaked in 0.5 M acetic acid, washed with PBS, incubated for 24 h in 0.01% gelatin (Sigma-Aldrich), and air dried. CXCL12 and fibrinogen (Sigma-Aldrich) in M199, containing 1% FCS, were seeded in the lower compartment, and 50 μl of HUVECs (2×10^6/ml) was added to the upper compartment. After 6 h of incubation at 37°C, the upper surface of the filter was scraped with a rubber policeman. The filters were fixed and stained, and five oil immersion fields (lower surface) were counted after coding samples.

Northern Blot Analysis. Cells were cultured in medium alone or supplemented with the indicated agents and total RNA was purified as described previously (10). 10 μg of total RNA from each sample was electrophoresed under denaturing conditions, blotted onto Nytran membranes (Schleicher & Schuell), and cross-linked by UV irradiation. Membranes were prehybridized at 42°C in hybrisol (Oncor, Inc.) and hybridized overnight with 10^6 cpm/ml of ^{32}P-labeled probe. Membranes were washed three times at room temperature for 10 min in 0.2× SSC (1× SSC = 0.15 M NaCl, 0.015 M of sodium citrate, pH 7.0), 0.1% SDS, and twice at 60°C for 20 min in 0.2× SSC, 0.1% SDS before being autoradiographed using films and intensifier screens at −80°C (XAR-5; Kodak). cDNAs were labeled by random priming using a commercial kit (Boehringer) and α-[^{32}P]dCTP (3,000 Ci/mmol; Amersham Biosciences). CCR1 and CCR5 cDNAs were obtained as described previously (11). CXCR4 cDNA was provided by T.N.C. Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland). Densitometric analysis was performed with a scanning densitometer (model GS300; Hoefer Scientific Instruments).

Transient Transfection. DNA plasmids were prepared using a commercially available kit (Endofree Maxi-Prep; QIAGEN). Transfections were performed using effectene transfection reagents (QIAGEN) according to the manufacturer's instructions. Cells were seeded at a concentration of 5×10^4 per well in 48-well plates the day before transfection. 24 h after transfection, reagents were removed, and cells were allowed to recover for 8 h before being treated for 16–24 h. Cotransfection experiments were performed using a 1:1 ratio between the reporter plasmid and HIF-1α expression vector. Luciferase reporter assays were performed in 96-well optiplates (Packard Instrument Co.) using a luciferase assay system (Promega) according to the manufacturer's instructions. Results were normalized for the protein content using the protein assay (Bio-Rad Laboratories). Reporter gene assay pGL–Hyp responsive element (HRE) plasmid contains three copies of the canonical HRE (5′-GTGAC-TACGTGCTGCCTAG-3′) from the inducible nitric oxide synthase promoter (26). pCXCR4 plasmid, containing a 2.6-kb fragment from the human CXCR4 promoter upstream of the luciferase reporter gene, was obtained from A.J. Caruz (Universidad de Jaen, Madrid, Spain; reference 27). HIF-1 α (ODD−) expression vector was obtained from E. Huang (Brigham and Women's Hospital, Harvard Medical School, Boston, MA; reference 28). The pCMV(HA)–HIF-1α expression vector was obtained from D. Livingston (Dana Farber Cancer Institute, Boston, MA).

Chromatin Immunoprecipitation Assay (ChIP). ChIP assays was performed in CAOV3 cells, transiently transfected with the p(HA)HIF-1α expression vector, and performed as described previously (29). In brief, 4×10^6 cells were fixed by adding directly to the medium formaldehyde (formaldehyde from a 37% formaldehyde/10% methanol stock; Calbiochem) to a final concentration of 1%. After 10 min, ice-cold PBS was immediately added, plates were transferred on ice and washed extensively with PBS, and cells were collected. After centrifugation, cells were lysed for 5 min in L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, and 10% Glycerol) supplemented with protease inhibitors. Nuclei were pelleted at 3,000 rpm in microfuge and resuspended in L2 buffer (50 mM Tris, pH 8.0, 1% SDS, and 5 mM EDTA). Chromatin was sheared by sonication (5×10 s at one fifth of the maximum potency in a Sonics vibracell VC13 equipped with a 3-mm tip), centrifuged to pellet debris, and diluted ten times in dilution buffer (50 mM Tris, pH 8.0, 0.5% NP-40, 0.2 M NaCl, and 0.5 mM EDTA). Extracts were precleared for 2 h with 80 μl of a 50% suspension of salmon sperm DNA–saturated protein A. Immunoprecipitations were performed at 4°C overnight with 2 μg of polyclonal anti–human hemagglutin antibody (Santa Cruz Biotechnology, Inc.). Immune complexes were collected with salmon sperm DNA–saturated protein A, and washed three times (5 min each) with high salt buffer (washing buffer: 20 mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 2 mM EDTA, and 500 mM NaCl), two times with a 0.5 M LiCl buffer, and three times with low salt buffer (1× TE). Immune complexes were extracted in 1× TE containing 2% SDS and protein; DNA cross-links were reverted by heating at 65°C for 6 h. After proteinase K digestion (100 μg, 1–2 h at 50°C), DNA was extracted by phenol, chloroform, and ethanol precipitated. Approximately 1/20 of the immunoprecipitated DNA was used in each PCR. Sequences of promoter-specific primers included the CXCR4 promoter region −1860 to −1578 as follows: CXCR4, sense, 5′-TCGTGCCAAAGCTTGTCCCTG-3′; and CXCR4, antisense, 5′-GCGGTAACCAATTCGCGAATAGTGC-3′.

Real-time PCR. Total RNA from MEFs, 786.0, and WT2 cells was obtained using an RNA mini kit (QIAGEN). RT-PCR was performed using a RT-PCR kit (PE Biosystems) as described previously (30). To measure the human vascular endothelial growth factor (VEGF), human CXCR4 and mouse CXCR4 expression real-time PCR was performed using a sequence detector (ABI-Prism, model 7700; Applied Biosystems). The following primers were used: human CXCR4, forward, 5′-GCA-TGACGGACAAGTACAGGCT-3′, reverse, 5′-AAAGTAC-CAGTTTGCCACGGC-3′; and mouse CXCR4, forward, 5′-TTGTCCACGCCACCAACAGTCA-3′, reverse, 5′-TGAAA-CACCACCATCCACAGGC-3′. Detection of 18S rRNA, used as internal control, was performed using premixed reagents from Applied Biosystems. Detection of VEGF and 18S rRNA was performed using a PCR master mix (TaqMan Universal; Applied

Biosystems) and CXCR4 detection was also performed using a PCR master mix (SyBr Green; Applied Biosystems). Detection of SDF-1/CCL12 expression by the MCF7 and CAOV3 cell lines was performed by using a PCR master mix (SyBr Green; Applied Biosystem), and the following primers were used: human CXCL12, forward, 5′-ACACTCCAAACTGTGCCCTTCA-3′; and human CXCL12, reverse, 5′-CCACGTCTTTGC-CCTTTCATC-3′.

Laser Confocal Microscopic Analysis of CXCR4 Expression. Cells growing on sterile coverslips were washed with PBS after 16-h incubation in Hyp or Norm conditions as indicated in the text. Non-specific sites were blocked by 10-min incubation at room temperature with wash buffer containing 0.9% wt/vol sodium chloride, 1% vol/vol human serum, and 0.02% wt/vol sodium azide. Thereafter, the cells were stained with mouse anti–human CXCR4 antibodies (BD Biosciences) at a dilution of 1:20 for 30 min at room temperature. The cells were washed twice with 2 ml of wash buffer and incubated with goat F(ab′)2 anti–mouse Ig-FITC (human adsorbed) at a dilution of 1:20 for another 30 min at room temperature. After two changes in wash buffer, the cells were fixed in 4% wt/vol paraformaldehyde for 15 min, and the coverslips were mounted on glass slides for microscopy. Images were visualized using a system with differential interference contrast (FV500; Olympus).

Results

Hyp-increased CXCR4 Expression in Mononuclear Phagocytes. As shown in Fig. 1 A, human monocytes cultured in Hyp, for periods of 4 and 6 h, showed a strong increase in the expression of CXCR4 mRNA, as compared with normoxia (Norm)-cultured monocytes (20% oxygen). Hyp-mediated up-regulation of CXCR4 mRNA was still present at 16 h. In Hyp, a slight increase was also observed for CCR1 mRNA expression, whereas CCR5 mRNA

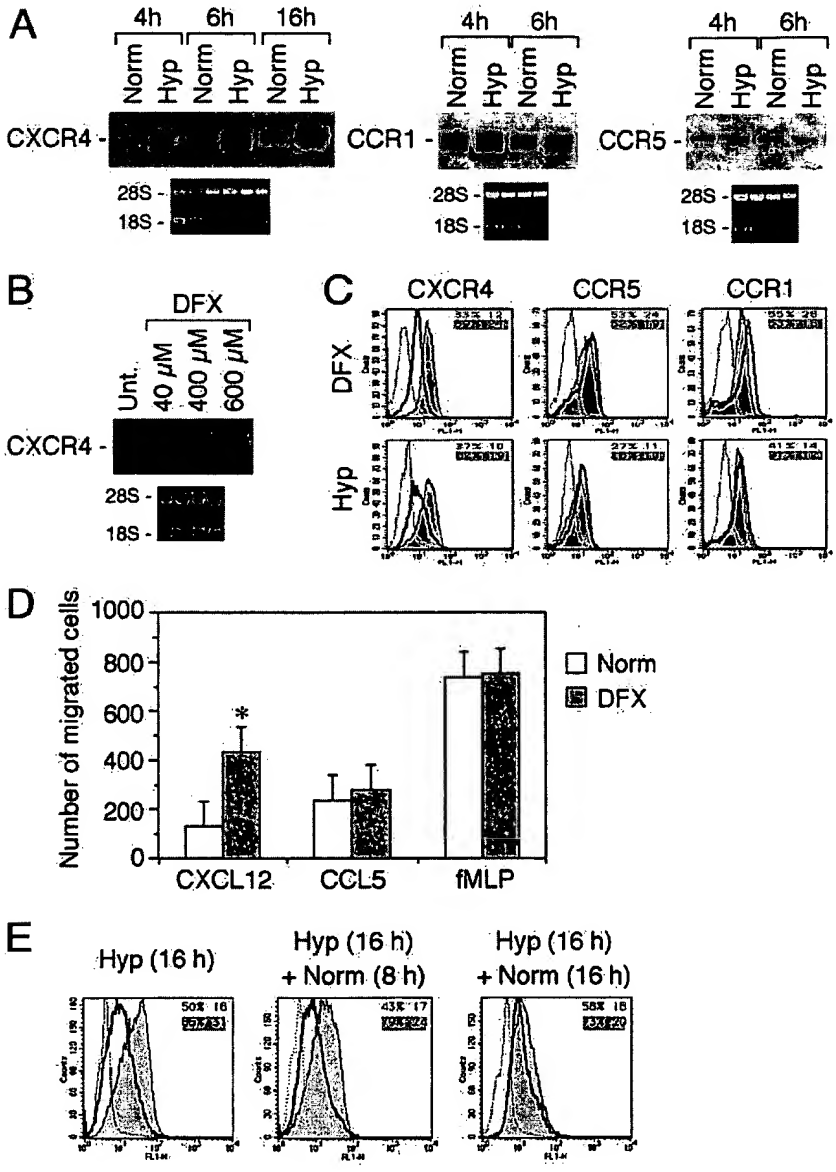


Figure 1. Effect of hypoxia (Hyp) on CXCR4 expression by fresh human monocytes. (A) Fresh human monocytes obtained from peripheral blood of healthy donors were cultured for different times in normoxia (Norm) or hypoxic (Hyp) conditions, as indicated. Total RNA was analyzed by Northern blot for CXCR4, CCR1, and CCR5 mRNA expression. (B) Total RNA from fresh human monocytes cultured for 4 h in the presence of increasing concentration of DFX was analyzed by Northern blot. (C) Hyp- and DFX-induced CXCR4 surface expression in fresh human monocytes. Cells were cultured for 16 h in the indicated conditions and analyzed for CXCR4 surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti–human CXCR4. The results are representative of three independent experiments. (dotted line) Irrelevant antibody. (continuous line) Norm. (shaded region) Hyp or DFX. (D) Effect of DFX on the chemotactic response of monocytes to 100 ng/ml CXCL12, 100 ng/ml CCL5, and 10^{-8} M FMLP. Cells were cultured for 16 h in the presence of 400 μM DFX. Migration of monocytes was assayed by chemotaxis microchamber technique. Results are mean ± SD of five experiments. *, $P < 0.05$ versus cells cultured in normoxic conditions (paired Student's *t* test). (E) Effect of reoxygenation on CXCR4 surface expression. Fresh human monocytes were cultured for 16 h in Hyp and subsequently reexposed to Norm for the indicated times. CXCR4 surface expression was determined by flow cytometry.

level was unaffected. Moreover, Hyp down-regulates responsiveness of monocytes to CCR2 agonists (4, 5). Therefore, we focused our attention on the CXCR4 receptor. The iron chelator DFX is recognized as an Hyp-mimicking compound (30). Incubation of fresh human monocytes for 4 h in the presence of DFX resulted in a dose-dependent increase of the CXCR4 mRNA levels (Fig. 1 B). Together, these results show that CXCR4 mRNA expression is selectively controlled by changes in oxygen levels. Modulation of CXCR4 by Hyp was confirmed at the protein level by flow cytometry. To evaluate the functional effects of Hyp on CXCR4, monocytes were incubated in hypoxic conditions and CXCR4 surface expression was determined. As shown in Fig. 1 C, exposure of monocytes for 16 h to Hyp resulted in a strong increase of CXCR4 surface expression. Similarly, cells incubated in the presence of 400 μM DFX for the same duration showed a significant increase of CXCR4 surface expression. In contrast, neither Hyp nor DFX treatment affected CCR1 and CCR5 surface expression. The observed DFX-dependent increase of CXCR4 surface expression was paralleled by a rise in the number of monocytes migrating in response to CXCL12 (Fig. 1 D). The increased chemotactic responsiveness toward CXCL12 was specific, whereas both CCL5 (regulated on activation normal T cell expressed and secreted) and FMLP-induced migration were not significantly affected. Thus, in human monocytes, low oxygen conditions result in a specific up-regulation of the CXCR4 expression and function. As it was important to assess the reversibility of Hyp on the induction of CXCR4 expression, we determined the effect of reoxygenation on the levels of CXCR4 surface expression in fresh human monocytes. As shown in Fig. 1 E, after culture in hypoxic conditions for 16 h and reoxygenation, monocytes retained high levels of CXCR4 for 8 h. Higher levels than control were still present at 16 h, and returned to baseline by 24 h (unpublished data).

Monocytes differentiate into macrophages during infiltration of tissues. As tissues are characterized by lower oxygen concentrations in comparison with peripheral circulation (1), it was important to determine whether CXCR4 up-regulation in response to low oxygen conditions could be applied also to macrophages. CXCR4 expression in Hyp was determined in fresh human monocytes, in vitro–differentiated MDMs, and TAMs obtained from the ascitic fluid of human ovarian carcinoma. Cells were cultured for 4 h in Norm or Hyp, and total RNA was analyzed for CXCR4 mRNA expression by Northern blot. As shown in Fig. 2, hypoxic conditions resulted in a strong up-regulation of the CXCR4 mRNA levels in all the three cell populations (Fig. 2 A). These results were confirmed by laser confocal microscopy Fig. 2 (B and C), wherein Hyp induced a strong up-regulation of CXCR4 surface expression, in both MDMs and TAMs. Clearly, Hyp controls CXCR4 expression in mononuclear phagocytes at different stages of differentiation.

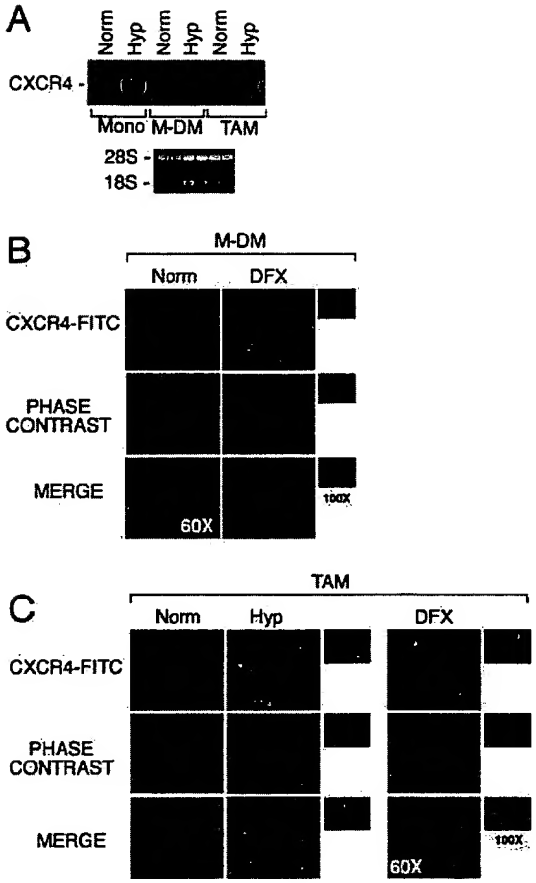


Figure 2. Effect of Hyp on CXCR4 expression by fresh human monocytes (mono), monocyte-derived macrophages (MDMs), and tumor-associated macrophages (TAMs). (A) Cells were cultured for 4 h in Hyp, and total RNA was analyzed by Northern blot for CXCR4 mRNA expression. MDM (B) and TAMs from ascitic fluid of human ovarian cancer (C) were cultured for 16 h in Norm, Hyp, or in the presence of 400 μM DFX, as indicated. CXCR4 surface staining was performed and detected by laser confocal microscopy. Staining with both isotype-matched control antibodies was done for all samples (not depicted).

Hyp-increased CXCR4 Expression in HUVECs. CXCL12 is a potent chemoattractant for endothelial cells of different origin and participates in angiogenesis (31, 32). We investigated the effect of Hyp on CXCR4 expression by endothelial cells. As shown in Fig. 3, HUVECs, cultured in Hyp or in the presence of 400 μM DFX for 4–16 h, showed increased CXCR4 mRNA expression, as compared with normoxic conditions. VEGF gene expression is induced by Hyp (1) and served as an internal control. As expected, VEGF mRNA levels were up-regulated either by Hyp or DFX. Next, CXCR4 surface expression was analyzed by laser confocal microscopy. As shown in Fig. 3 B, after Hyp and DFX treatment, CXCR4 surface expression in endothelial cells was strongly increased. Endothelial cell recruitment represents an initial step of the process of angiogenesis. HUVECs cultured in Norm or Hyp were tested for their capability to migrate in response to CXCL12. As shown in Fig. 3 C, the number of migrated

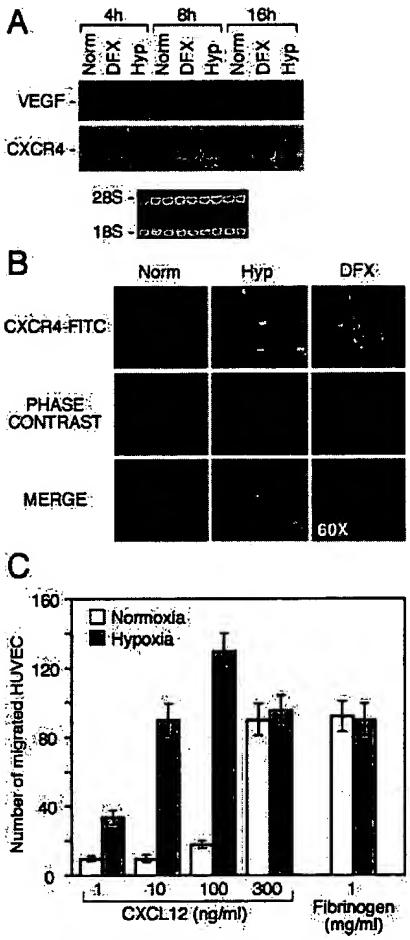


Figure 3. Effect of Hyp on CXCR4 expression by human endothelial venules (HUVECs). (A) Cells were cultured for 4, 8, and 16 h under Norm, Hyp, or in the presence of 400 μM DFX, respectively. Thereafter, total RNA was analyzed by Northern blot for CXCR4 and VEGF mRNAs expression. (B) HUVECs were cultured for 16 h in Norm, Hyp, and in the presence of 400 μM DFX, as indicated. CXCR4 surface staining was performed and detected by laser confocal microscopy. (C) Effect of Hyp on the chemotactic response of HUVECs to CXCL12. Cells were cultured for 16 h in hypoxic conditions. Fibrinogen was used as a reference attractant. Results are mean ± SD of three experiments.

HUVECs in response to CXCL12 was significantly higher in hypoxic conditions, at concentrations ranging from 1 to 100 ng/ml. CXCL12 was ~100-fold more effective at eliciting HUVEC migration under Hyp than under normoxic conditions. Migration elicited by fibrinogen, used as a control, was not affected.

Hyp-increased CXCR4 Expression in Cancer Cells. Chemokine receptors may act as molecular tools exploited by cancer cells to metastasize to target organs (33). In particular, CXCR4 was shown to play a major role in the migration of breast cancer cells from the primary tumor to secondary metastatic sites, such as lung, liver, and bone. As solid tumors are often characterized by the presence of necrotic areas with low oxygen tension, we reasoned that Hyp may be a potential mechanism that up-regulates expression of chemokine receptors in cancer cells localized in poorly avascularized and oxygenated areas of tumors. To test this hypothesis, we investigated the level of CXCR4 mRNA expression in response to Hyp in the ovarian cancer cell line CAOV3 (Fig. 4). As shown in Fig. 4 A, CAOV3 cells showed up-regulation of CXCR4 mRNA after 4 h of culture under hypoxic conditions, which was paralleled by a significant increase in the surface expression of this receptor, as demonstrated by cytofluorimetric analysis (Fig. 4 B). The Hyp-induced expression of CXCR4 in the CAOV3 cells correlated with an increased migration of these cells toward CXCL12 in the chemotaxis assay (Fig. 4 C). Similar results were obtained with the MCF-7 breast cancer cell line (unpublished data). Fig. 4 D shows the effect of Hyp on CXCL12 expression by the same cancer cell lines MCF7 and CAOV3.

CXCR4 expression by cancer cells may play an important role in the metastatic process (33). The process of metastasis formation includes the exit of cancer cells from the primary tumor site and their entrance into the circulation, where the oxygen levels will markedly increase, relative to those present in the tumor microenvironment. To assess the impact that the exposure of cancer cells to reoxygenation had on the CXCR4 surface expression, CAOV3 cells were cultured in hypoxic conditions for 16 h and subsequently exposed to Norm for up to 14 h. As shown in Fig. 4 E, Hyp-increased CXCR4 surface expression in CAOV3 cells was retained after reoxygenation for 14 h and returned to baseline by 24 h (not depicted).

Involvement of HIF-1α in Hyp-induced Expression of CXCR4. HIF-1 is a key regulator of the transcriptional response to Hyp. To investigate the role of HIF-1 in the hypoxic induction of CXCR4, MEFs from WT or deficient animals for the α subunit of HIF-1 were incubated under normoxic or hypoxic conditions for 6 h, and total RNA was tested for VEGF and CXCR4 mRNA levels by real-time PCR. As shown in Fig. 5 A, MEFs from WT animals, but not from HIF-1α KO mice, expressed 3.5-fold-higher levels of VEGF mRNA when incubated under hypoxic conditions relative to levels expressed in normoxic conditions. Likewise, MEFs from WT mice expressed 2.5-fold-higher levels of CXCR4 mRNA when cultured under hypoxic conditions relative to normoxic conditions. In contrast, in MEFs from HIF-1$\alpha^{-/-}$ mice, the hypoxic induction of CXCR4 mRNA was no longer apparent. Furthermore, DFX also induced VEGF and CXCR4 mRNA in HIF-1 WT cells (6.5- and 7.2-fold, respectively) but not in HIF-1$\alpha^{-/-}$ cells (Fig. 5 B). These data suggest a role for HIF-1 in the induction of CXCR4 mRNA expression by Hyp.

The von Hippel–Lindau tumor suppressor protein (VHL) is involved in the degradation of HIF-α; mutations of VHLs are associated with high levels of HIF-1α protein and transcriptional activity (34, 35). Therefore, we tested the expression of CXCR4 mRNA in the renal cancer cell line 786.0 (VHL mutated; reference 36) and the WT2 cell line (in which a WT VHL has been reintroduced). These cells have been extensively used in several laboratories as

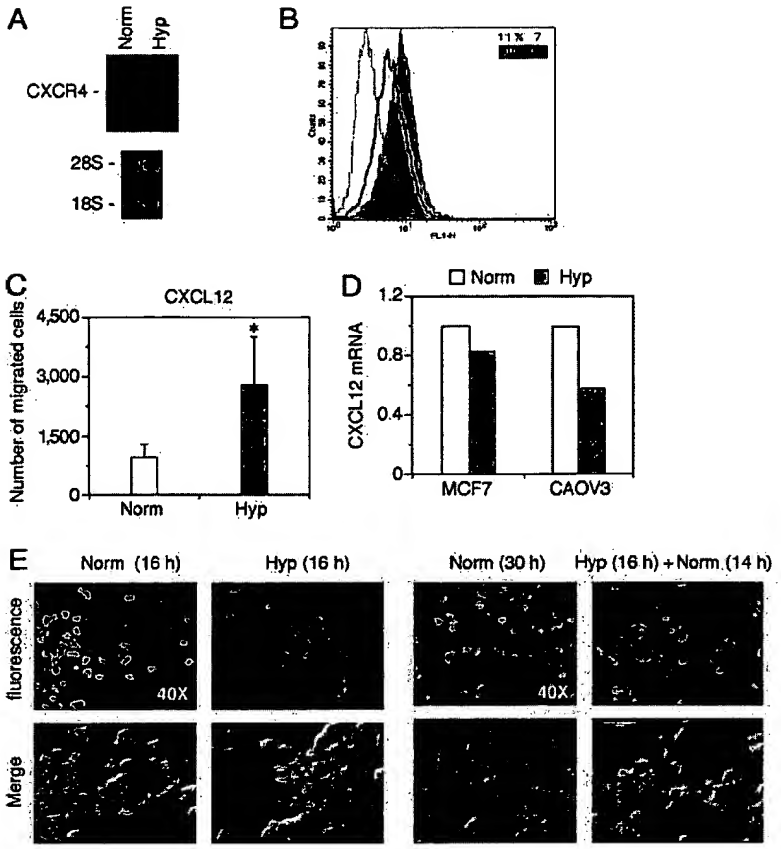


Figure 4. Effects of Hyp on CXCR4 expression by cancer cells. The ovarian cancer cell line CAOV3 was cultured in normoxic or hypoxic conditions and analyzed respectively for both CXCR4 mRNA expression (A) and CXCR4 surface expression (B). (A) cells were cultured for 4 h in normoxia (Norm) or hypoxic (Hyp) conditions, as indicated. Total RNA was analyzed by Northern blot for CXCR4. (B) Cells were cultured for 16 h in Norm or hypoxic (Hyp) conditions. After this period, CXCR4 surface expression was determined by flow cytometry. (dotted line) Irrelevant antibody. (continuous line) Norm. (shaded region) Hyp. (C) Effect of Hyp on the chemotactic response of CAOV3 ovarian cancer cells to 100 ng/ml CXCL12. Cells were incubated overnight in Hyp condition and migration determined by Transwells, as described in Materials and Methods. Results are mean ± SD of three experiments. *, $P < 0.05$ versus cells cultured in normoxic conditions (Paired Student's t test). (D) Effects of Hyp on CXCL12 mRNA expression by MCF-7 and CAOV3 cells. Cells were incubated for 4 h in Norm and Hyp as indicated, and CXCR4 gene expression was next determined by real-time PCR. Results are representative of two independent experiments. Values are expressed as fold increases relative to the reference sample (Norm). (E) Sustained Hyp-induced CXCR4 expression upon reoxygenation. CAOV3 cells were cultured under Norm or Hyp conditions for 16 h. Thereafter, the cells were exposed to Norm for a further 14 h and stained for CXCR4 expression. The figure shows a representative field using confocal microscopy. CAOV3 cells stained for CXCR4 (blue fluorescence) and nuclei (red fluorescence). The bottom panels in each group shows phase-contrast images merged with fluorescence readings.

prototype in which high levels of HIF-1 activity are associated with increased levels of expression of HIF-1–inducible genes (23). As reported previously (37), 786.0 cells expressed high constitutive levels of VEGF mRNA that were significantly lower (>60%) in WT2 cells (Fig. 5 C). Accordingly, 786.0 cells expressed higher levels of CXCR4 mRNA relative to WT2 cells, in which we observed >65% reduction in three independent experiments. These data again are consistent with the involvement of HIF-1 in the hypoxic regulation of CXCR4 mRNA expression. We investigated the role of HIF-1 in the transcriptional activation of CXCR4 promoter. MCF-7 breast carcinoma cells, in which Hyp augments expression of CXCR4 mRNA (unpublished data), were transiently transfected with a plasmids containing a 2.6-kb fragment of the CXCR4 promoter linked to the luciferase reporter gene and incubated under normoxic or hypoxic conditions for 24 h. As shown in Fig. 5 D, Hyp induced fourfold higher levels of luciferase expression relative to cells cultured under normoxic conditions. Interestingly, cotransfection of a HIF-1α expression vector significantly increased luciferase expression driven by the 2.6-kb CXCR4 promoter under normoxic conditions (approximately fourfold relative to control). Hyp further increased luciferase expression induced by HIF-1α cotransfection up to ninefold compared to untreated normoxic cells. Experiments conducted in parallel using a plasmid containing three copies of a canonical Hyp-responsive element upstream of the luciferase reporter gene showed a similar trend of luciferase expression, although levels of induction were substantially higher.

To obtain direct evidence for the interaction between HIF-1α and the CXCR4 promoter, we used the ChIP assay to measure the HIF-1α recruitment to the CXCR4 promoter. CAOV3 cells, either cultured in Norm or Hyp for different times, were fixed in formaldehyde, and subsequently analyzed by ChIP. Although no interaction between HIF-1α and the CXCR4 promoter was observed in Norm, recruitment of HIF-1α to the CXCR4 promoter was clearly detected at 4 h after Hyp, in the promoter region −1860 to −1578 (Fig. 5 E). This result is consistent with the functional data obtained in transient transfection experiments (Fig. 5 D). Moreover, the HIF-1α inhibitor Topotecan (38), was able to prevent the Hyp-induced upregulation of CXCR4 in CAOV3 cells (unpublished data). Overall these data demonstrate the involvement of HIF-1α in the induction of CXCR4 promoter.

Effect of Hyp and DFX on CXCR4 mRNA Stability. To further investigate the mechanisms of Hyp action, we estimated its effects on CXCR4 mRNA stability (Fig. 6). Fresh human monocytes were cultured either in Norm or Hyp in the presence or absence of 1 μg/ml actinomycin D. Total RNA was extracted at different times as indicated. The mRNA decay observed in Hyp conditions was compared with the rate of mRNA degradation observed in

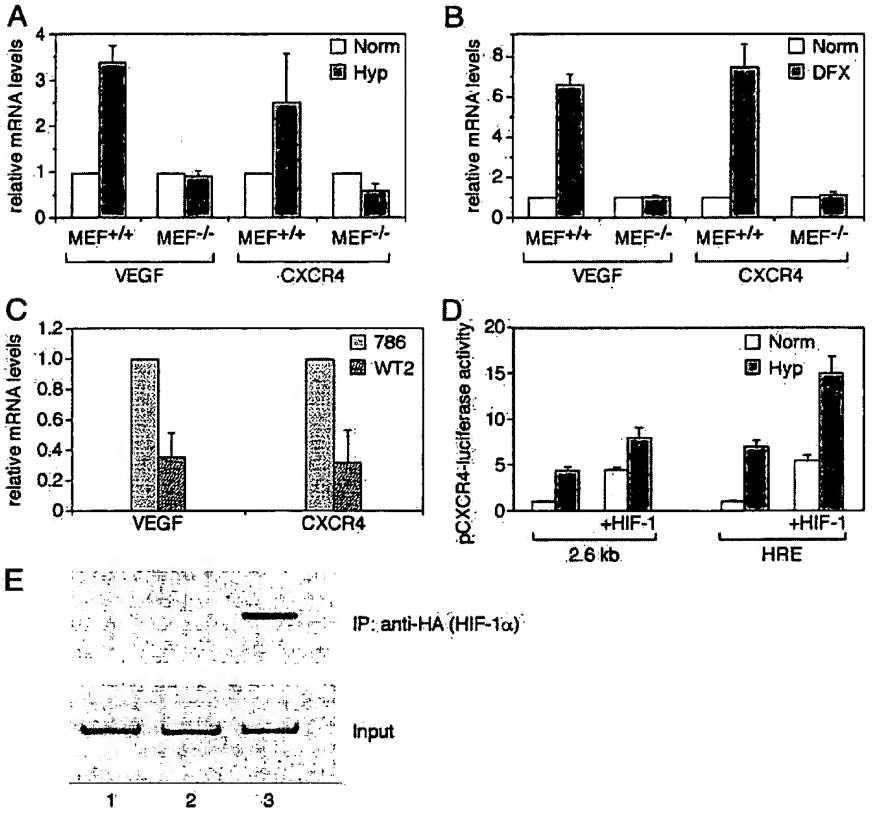


Figure 5. Role of HIF-1α in the regulation of CXCR4 gene expression. (A) Expression of CXCR4 in HIF-1α KO mouse embryo fibroblast. Mouse embryo fibroblast from wild type ($MEF^{+/+}$) or knockout for the α subunit of HIF-1 ($MEF^{-/-}$) were incubated under normoxic or hypoxic conditions for 6 h, and total RNA was tested for VEGF and CXCR4 mRNA levels by real-time PCR. (B) DFX was used as Hyp-inducing agent. Results are the average of three independent experiments. (C) Expression of CXCR4 in VHL WT and mutated renal carcinoma cells. Expression of CXCR4 and VEGF mRNAs was tested by real-time PCR in the renal cancer cell line 786.0 (VHL mutated) and WT2 (in which a WT VHL has been reintroduced). Results are the average of three independent experiments. (D) HIF-1–dependent transcriptional activation of CXCR4 promoter. MCF-7 breast carcinoma cells were transiently transfected with a plasmid containing a 2.6 kb fragment of the CXCR4 promoter linked to the luciferase reporter gene, with or without a HIF-1α expression vector. Cells were incubated under normoxic or hypoxic conditions for 24 h and evaluated for the luciferase activity. Results are the average of three independent experiments. (E) Hyp-induced HIF-1α recruitment to the CXCR4 promoter. CAOV3 cells transfected with the p(HA)HIF-1α plasmid were cultured for 4 h in normoxic or hypoxic conditions. ChIP was performed to investigate the recruitment of HIF-1α on the CXCR4 promoter. (lane 1) Untransfected. (lane 2) Norm. (lane 3) Hyp.

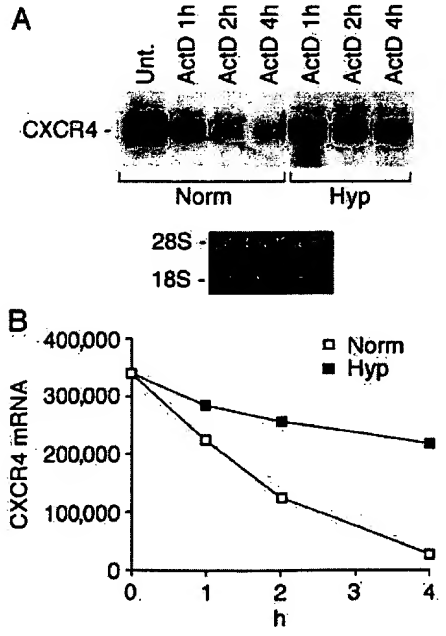


Figure 6. Stabilization of CXCR4 mRNA by Hyp. (A) Fresh human monocytes were cultured for 4 h under Norm or Hyp, and in the presence or absence of 1 μg/ml actinomycin D (ActD). Thereafter, total RNA was extracted at different times as indicated and analyzed by Northern blot for CXCR4 mRNA expression. (B) Densitometric analysis: CXCR4 mRNA levels are expressed as arbitrary units. In this experiment, the basal CXCR4 mRNA level appears higher compared with other experiments, as we exposed the film for a longer period for better visualization of the blot.

normoxic conditions. The results indicate that Hyp increased CXCR4 mRNA stability, suggesting that Hyp-induced CXCR4 mRNA expression relies on both transcriptional and posttranscriptional mechanisms.

Discussion

Regulation of cell migration by changes in oxygen availability is a central event during the organization of host response in inflammatory and neoplastic diseases as it may influence leukocyte recruitment and activation, angiogenesis, and metastasis formation (16). Here, we report that Hyp mediates selective up-regulation of CXCR4 in different cell types, including mononuclear phagocytes (monocytes, MDMs, and TAMs), endothelial cells, and cancer cells, and demonstrate that oxygen levels act as an important regulator of CXCR4 receptor expression. Our data also indicate

that HIF-1 activation is involved in the Hyp-dependent up-regulation of CXCR4 expression and that the Hyp–HIF-1–CXCR4 circuit may participate in pathophysiological mechanisms under several conditions, ranging from inflammation to tumor angiogenesis and metastasis.

In contrast to standard cell culture conditions, characterized by 20% oxygen concentration, cells in the human body are exposed to much lower oxygen concentrations, ranging from 16% in the pulmonary alveoli to <6% in most other organs of the body. Moreover, oxygen concentration may even drop to extremely low concentrations, close to anoxia, in the presence of altered vascularization as observed at pathological sites such as tumors (1). As selective accumulation of leukocyte subpopulations is the hallmark in allergy, inflammation, and tumors (18), it was important to investigate how leukocyte recruitment is affected by changes in oxygen tension. We first observed that in response to Hyp monocytes and MDM increase, CXCR4 expression and function, as assessed by surface expression and chemotactic responsiveness to its specific ligand CXCL12. Thus, dynamic regulation of the chemotactic responsiveness of monocytes/macrophages may represent a feature of the pathophysiology of inflammatory diseases associated with Hyp. Interestingly, Hyp is present in the joint microenvironment, because articular cartilage is an avascular tissue that functions at lower oxygen tension than do most tissues. Moreover, in the setting of diseases such as rheumatoid arthritis and osteoarthritis, in which macrophages promote perpetuation of chronic inflammation (39), a further decrease in synovial fluid oxygen tension may occur (40, 41).

In solid tumors, TAMs represent a prominent component of the mononuclear leukocyte population, which displays an ambivalent relationship with tumors (the "macrophage balance hypothesis"; reference 17). Interestingly, TAMs preferentially localize at the tumor–host tissue interface, in regions often associated with low oxygen tensions. Several lines of evidence also indicate that chemokines play a pivotal role in the recruitment of monocytes in neoplastic tissues (17, 18, 42) and a variety of chemokines have been detected as products of cancer cells or tumor stromal elements. In particular, CCL2 was proposed as tumor-derived chemotactic factors, which play a major role in the recruitment of macrophages at the tumor site (18). Receptor expression is a crucial determinant of the spectrum of action of chemokines (18). It was reported that the capacity of monocytes/macrophages to migrate in response to CCL2 is decreased in low oxygen conditions (4, 5). Thus, our observation of Hyp-mediated up-regulation of CXCR4 expression in TAMs may indicate that in regions associated with oxygen decrease, a dynamic change of their receptor profile occurs, with up-regulation of functional CXCR4. In support of this hypothesis, Cramer et al. have recently shown that myeloid cell infiltration in vivo is dependent on the presence of an intact HIF-1α subunit, suggesting that oxygen gradient may be a critical factor for myeloid cells' migration in inflammatory sites (43). A relay of distinct chemokine–chemokine receptor interactions may regulate initial recruitment, tissue infiltration in hypoxic areas, and in neoplastic and non-neoplastic inflammatory sites in a multistep navigation process (44).

Angiogenesis is a prerequisite for the expansion of solid tumors and is often activated during the early, preneoplastic stages of tumor development (45, 46). Tumor angiogenesis is controlled by a number of positive and negative regulators produced by cancer cells and tumor-associated leukocytes. A number of molecules with possible impact on angiogenesis have been shown to be expressed by macrophages in low oxygen conditions, such as VEGF, TNF-α, bFGF, and CXCL8 (47). The contribution of chemokines toward angiogenesis is currently a focus of intensive investigation (48). Strikingly, it was recently reported that CXCL12 acts as a potent chemoattractant for endothelial cells of different origins bearing CXCR4 and is a participant in angiogenesis that is regulated at the receptor level by VEGF and bFGF (31, 49–51). In agreement with these observations, we observed an increased chemotactic responsiveness of HUVECs toward CXCL12, which may well be part of the Hyp-induced angiogenic program. Hyp is a well-recognized pathophysiological condition for the induction of angiogenic factors, including but not limited to VEGF (16). In agreement with these observations, our data suggest that the angiogenic program established by Hyp may rely also on the increased expression of CXCR4 by different cellular components in the tumor microenvironment, including endothelial cells, tumor cells, and TAMs.

The involvement of CXCR4 in cancer metastasis has been proposed by Muller and colleagues, who showed that this receptor and its ligand (CXCL12) together govern the pattern of breast cancer metastasis in a mouse model (33, 52). This observation is in support of the "chemoattraction" theory of metastasis, which holds that organ-specific attractant molecules stimulate the migrating tumor cells to invade the walls in blood vessels and enter the organs. However, this remarkable observation does not clarify the mechanisms of selection by which cancer cells became CXCR4 positive. Our observation that the levels of CXCR4 surface expression induced by Hyp are sustained for several hours after reoxygenation is consistent with the idea that this pathway may confer metastatic potential to cancer cells. Indeed, it was described in in vivo models of metastasis that after entering the circulation, the majority of cancer cells home to target organs in a timeframe ranging from 1 to 24 h (53). We propose that in solid tumors, in addition to genetic alterations such as mutation of VHL, PTEN, or p53 genes that are associated with increased levels of HIF-1 transcriptional activity, microenvironmental Hyp may increase CXCR4 expression and the metastatic potential of cancer cells. In line with this hypothesis, recent evidence has been provided that invasive cancer phenotype is associated with Hyp and/or HIF-1α overexpression (54). In agreement with the data and concepts described here, by using immunohistochemical analysis, we have observed that ductal carcinoma cells from breast tissue located in areas of intratumoral necrosis display nuclear HIF-1α expression and high levels of CXCR4 (unpublished data).

HIF-1 activates transcription of genes that mediate adaptive responses to reduced oxygen availability and a number of HIF-1–regulated genes have been identified, whose products play key roles in angiogenesis, vascular reactivity and remodelling, and glucose and energy metabolism (1). HIF-1 is a heterodimer composed of a HIF-1β subunit that is constitutively expressed and a HIF-1α subunit that is rapidly degraded by ubiquitination via the proteasomal pathway, a process that is inhibited under hypoxic conditions. Oxygen-regulated destruction of HIF-1α requires the von Hippel–Lindau tumor suppressor protein (pVHL) (34, 35). pVHL acts as the recognition component of a ubiquitin E3 ligase complex, which binds HIF-1α, and loss of pVHL function results in constitutive activation of the hypoxic response (35, 55). A role for HIF-1 in the regulation of CXCR4 mRNA expression is suggested by the following findings: (a) mouse embryonal fibroblast lacking the α subunit of HIF-1 had impaired hypoxic induction of CXCR4 mRNA; (b) CXCR4 mRNA was differentially expressed in the renal cancer cell lines 786.0 and WT2 bearing a VHL-mutated and WT phenotype, respectively; (c) Hyp or cotransfection of a HIF-1α expression vector–induced transcriptional activation of a 2.6-kb CXCR4 promoter luciferase reporter construct; (d) ChIP analysis demonstrated that after Hyp, HIF-1α is specifically recruited to the CXCR4 promoter, in the nucleotide region −1860 to −1578; and (e) the HIF-1 inhibitor Topotecan inhibited the Hyp-induced expression of CXCR4 in CAOV3 cells. A sequence homology search of the region −1860 to −1578 of the CXCR4 promoter revealed the presence of a putative HIF-1 binding site at position −1725 (5′-GCGTG-3′). Ongoing studies in the laboratory will provide a full biochemical and functional characterization of the HRE. In addition to transcriptional activation, we found that Hyp further contributes to increased CXCR4 gene expression by stabilization of CXCR4 transcripts, suggesting that Hyp-regulated RNA binding factors may interact with and stabilize the CXCR4 mRNA at the post-transcriptional level.

Our data identify the Hyp–HIF-1–CXCR4 pathway as a relevant molecular circuit in the functional tuning of the chemokine system. The validity of this observation in different cell types (mononuclear phagocytes, HUVECs, fibroblasts, and cancer cells), consistent with the virtually universal expression of the Hyp–HIF-1 pathway in mammalian cells, argues in favor of its potential involvement in the pathophysiology of diverse conditions. In a multistep navigation process, the Hyp–HIF-1α–CXCR4 pathway may regulate trafficking and localization in hypoxic tissues and represents a target for novel therapeutic strategies.

We thank Dr. P. Allavena for critical revision of the manuscript. We thank M. Sironi, N. Polentarutti, and G. Balconi for technical support.

This work was supported by European Community and the Ministero Istruzione Università e Ricerca, Fondi per gli Investimenti della Ricerca di Base project. The contribution of the Italian Association for Cancer Research is acknowledged. This work was supported by the scholarship in memory of Maria Teresa Pignatelli, by a fellowship from the Alfredo Leonardi Fund, and G.L. Pfieffer Foundation. This project has been funded in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organization imply endorsement by the U.S. government.

Submitted: 19 February 2003
Revised: 4 August 2003
Accepted: 16 September 2003

References

1. Semenza, G.L. 2001. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol. Med.* 7:345–350.
2. Semenza, G.L. 1999. Perspectives on oxygen sensing. *Cell.* 98:281–284.
3. Wang, G.L., B.H. Jiang, E.A. Rue, and G.L. Semenza. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc. Natl. Acad. Sci. USA.* 92:5510–5514.
4. Grimshaw, M.J., and F.R. Balkwill. 2001. Inhibition of monocyte and macrophage chemotaxis by hypoxia and inflammation–a potential mechanism. *Eur. J. Immunol.* 31:480–489.
5. Turner, L., C. Scotton, R. Negus, and F. Balkwill. 1999. Hypoxia inhibits macrophage migration. *Eur. J. Immunol.* 29: 2280–2287.
6. Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature.* 392:565–568.
7. Hedrick, J.A., and A. Zlotnik. 1996. Chemokines and lymphocyte biology. *Curr. Opin. Immunol.* 8:343–347.
8. Rollins, B.J. 1997. Chemokines. *Blood.* 90:909–928.
9. Murphy, P.M. 2002. International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol. Rev.* 54:227–229.
10. Sica, A., A. Saccani, A. Borsatti, C.A. Power, T.N. Wells, W. Luini, N. Polentarutti, S. Sozzani, and A. Mantovani. 1997. Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J. Exp. Med.* 185:969–974.
11. Sica, A., A. Saccani, B. Bottazzi, S. Bernasconi, P. Allavena, B. Gaetano, F. Fei, G. LaRosa, C. Scotton, F. Balkwill, and A. Mantovani. 2000. Defective expression of the monocyte chemotactic protein-1 receptor CCR2 in macrophages associated with human ovarian carcinoma. *J. Immunol.* 164:733–738.
12. Sica, A., A. Saccani, and A. Mantovani. 2002. Tumor-associated macrophages: a molecular perspective. *Int. Immunopharmacol.* 2:1045–1054.
13. Mantovani, A. 1999. The chemokine system: redundancy for robust outputs. *Immunol. Today.* 20:254–257.
14. Madjdpour, C., U.R. Jewell, S. Kneller, U. Ziegler, R. Schwendener, C. Booy, L. Klausli, T. Pasch, R.C. Schimmer, and B. Beck-Schimmer. 2003. Decreased alveolar oxygen induces lung inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 284:360–367.
15. Mapp, P.I., M.C. Grootveld, and D.R. Blake. 1995. Hypoxia, oxidative stress and rheumatoid arthritis. *Br. Med. Bull.*

51:419–436.
16. Semenza, G.L. 2002. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol. Med.* 8:S62–S67.
17. Mantovani, A., B. Bottazzi, F. Colotta, S. Sozzani, and L. Ruco. 1992. The origin and function of tumor-associated macrophages. *Immunol. Today.* 13:265–270.
18. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23:549–555.
19. Leek, R.D., C.E. Lewis, R. Whitehouse, M. Greenall, J. Clarke, and A.L. Harris. 1996. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res.* 56:4625–4629.
20. Leek, R.D., R.J. Landers, A.L. Harris, and C.E. Lewis. 1999. Necrosis correlates with high vascular density and focal macrophage infiltration with angiogenesis and prognosis in invasive carcinoma of the breast. *Br. J. Cancer.* 79:991–995.
21. Mantovani, A., V. Caprioli, P. Gritti, and F. Spreafico. 1977. Human mature macrophages mediate antybody-dependent cellular cytotoxicity on tumor cells. *Transplantation.* 24:291–293.
22. Romano, M., M. Sironi, C. Toniatti, N. Polentarutti, P. Fruscella, P. Ghezzi, R. Faggioni, W. Luini, V. van Hinsbergh, S. Sozzani, et al. 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity.* 6:315–325.
23. Ivanov, S.V., I. Kuzmin, M.H. Wei, S. Pack, L. Geil, B.E. Johnson, E.J. Stanbridge, and M.I. Lerman. 1998. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. *Proc. Natl. Acad. Sci. USA.* 95:12596–12601.
24. Scotton, C.J., J.L. Wilson, D. Milliken, G. Stamp, and F.R. Balkwill. 2001. Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res.* 61:4961–4965.
25. Colotta, F., F. Bussolino, N. Polentarutti, A. Guglielmetti, M. Sironi, E. Bocchietto, M. De Rossi, and A. Mantovani. 1993. Differential expression of the common b and specific a chains of the receptors for GM-CSF, IL-3, and IL-5 in endothelial cells. *Exp. Cell Res.* 206:311–317.
26. Melillo, G., T. Musso, A. Sica, L.S. Taylor, G.W. Cox, and L. Varesio. 1995. A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J. Exp. Med.* 182:1683–1693.
27. Caruz, A., M. Samsom, J.M. Alonso, J. Alcami, F. Baleux, J.L. Virelizier, M. Parmentier, and F. Arenzana-Seisdedos. 1998. Genomic organization and promoter characterization of human CXCR4 gene. *FEBS Lett.* 426:271–278.
28. Huang, E., J.M. Gu, M. Schau, and H.F. Bunn. 1998. Regulation of hypoxia-inducible factor 1 is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA.* 95:7987–7992.
29. Saccani, S., S. Pantano, and G. Natoli. 2002. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat. Immunol.* 3:69–75.
30. Wang, G.L., and G.L. Semenza. 1993. Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood.* 82:3610–3615.
31. Salcedo, R., K. Wasserman, H.A. Young, M.C. Grimm, O.M. Howard, M.R. Anver, H.K. Kleinman, W.J. Murphy, and J.J. Oppenheim. 1999. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: in vivo neovascularization induced by stromal-derived factor-1alpha. *Am. J. Pathol.* 154:1125–1135.
32. Nagasawa, T., S. Hirota, K. Tachibana, N. Takakura, S. Nishikawa, Y. Kitamura, N. Yoshida, H. Kikutani, and T. Kishimoto. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature.* 382:635–638.
33. Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, et al. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature.* 410:50–56.
34. Hon, W.C., M.I. Wilson, K. Harlos, T.D. Claridge, C.J. Schofield, C.W. Pugh, P.H. Maxwell, P.J. Ratcliffe, D.I. Stuart, and E.Y. Jones. 2002. Structural basis for the recognition of hydroxyproline in HIF-1 alpha by pVHL. *Nature.* 417:975–978.
35. Min, J.H., H. Yang, M. Ivan, F. Gertler, W.G. Jr. Kaelin, and N.P. Pavletich. 2002. Structure of an HIF-1alpha-pVHL complex: hydroxyproline recognition in signaling. *Science.* 296:1886–1889.
36. Lieubeau-Teillet, B., J. Rak, S. Jothy, O. Iliopoulos, W. Kaelin, and R.S. Kerbel. 1998. von Hippel-Lindau gene-mediated growth suppression and induction of differentiation in renal cell carcinoma cells grown as multicellular tumor spheroids. *Cancer Res.* 58:4957–4962.
37. Stratman, R., M. Krieg, R. Haas, and K.H. Plate. 1997. Putative control of angiogenesis in hemangioblastomas by the von Hippel–Lindau tumor supressor gene. *J. Neuropathol. Exp. Neurol.* 56:1142–1152.
38. Rapisarda, A., B. Uranchimeg, D.A. Scudiero, M. Selby, E.A. Sausville, R.H. Shoemaker, and G. Melillo. 2002. Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. *Cancer Res.* 62: 4316–4324.
39. Liew, F.Y., and I.B. McInnes. 2002. The role of innate mediators in inflammatory response. *Mol. Immunol.* 38:887–890.
40. Blake, D.R., P.G. Winyard, and R. Marok. 1994. The contribution of hypoxia-reperfusion injury to inflammatory synovitis: the influence of reactive oxygen intermediates on the transcriptional control of inflammation. *Ann. NY Acad. Sci.* 723:308–317.
41. Cernanec, J., F. Guilak, J.B. Weinberg, D.S. Pisetsky, and B. Fermor. 2002. Influence of hypoxia and reoxygenation on cytokine-induced production of proinflammatory mediators in articular cartilage. *Arthritis Rheum.* 46:968–975.
42. Gerard, C., and B.J. Rollins. 2001. Chemokines and disease. *Nat. Immunol.* 2:108–115.
43. Cramer, T., Y. Yamanishi, B.E. Clausen, I. Forster, R. Pawlinski, N. Mackman, V.H. Haase, R. Jaenisch, M. Corr, V. Nizet, et al. 2003. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell.* 112:645–657.
44. Foxman, E.F., J.J. Campbell, and E.C. Butcher. 1997. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J. Cell Biol.* 139:1349–1360.
45. Folkman, J. 1995. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* 1:27–31.
46. Hanahan, D., and J. Folkman. 1996. Patterns of emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell.* 86:353–364.
47. Crowther, M., N.J. Brown, E.T. Bishop, and C.E. Lewis. 2001. Microenvironmental influence on macrophage regulation of angiogenesis in wounds and malignant tumors. *J. Leu-*

koc. Biol. 70:478–490.
48. Hirani, N., F. Antonicelli, R.M. Strieter, M.S. Wiesener, P.J. Ratcliffe, C. Haslett, and S.C. Donnelly. 2001. The regulation of interleukin-8 by hypoxia in human macrophage–a potential role in the pathogenesis of the Acute Respiratory Distress Syndrome (ARDS). *Mol. Med.* 10:685–697.
49. Payne, A.S., and L.A. Cornelius. 2002. The role of chemokines in melanoma tumor growth and metastasis. *J. Invest. Dermatol.* 118:915–922.
50. Nagasawa, T. 2001. Role of chemokine SDF-1/PBSF and its receptor CXCR4 in blood vessel development. *Ann. NY Acad. Sci.* 947:112–115.
51. Strieter, R.M., P.J. Polverini, D.A. Arenberg, and S.L. Kunkel. 1995. The role of CXC chemokines as regulators of angiogenesis. *Shock.* 4:155–160.
52. Liotta L.A. 2001. An attractive force in metastasis. *Nature.* 410:24–25.
53. Aoudjit, F., E.F. Potworowski, and Y. St-Pierre. 1998. The metastatic characteristics of murine lymphoma cell lines in vivo are manifested after target organ invasion. *Blood.* 91: 623–629.
54. Krishnamachary, B., S. Berg-Dixon, B. Kelly, F. Agani, D. Feldser, G. Ferreira, N. Iyer, J. LaRusch, B. Pak, P. Taghavi, and G.L. Semenza. 2003. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. *Cancer Res.* 63:1138–1143.
55. Pugh, C.W., and P.J. Ratcliffe. 2003. The von Hippel-Lindau tumor suppressor, hypoxia-inducible factor-1 (HIF-1) degradation, and cancer pathogenesis. *Semin. Cancer Biol.* 13: 83–89.

Expansion of human SCID-repopulating cells under hypoxic conditions

Guénahel H. Danet,[1] Yi Pan,[2] Jennifer L. Luongo,[1] Dominique A. Bonnet,[3] and M. Celeste Simon[2]

[1]Division of Hematology-Oncology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA
[2]Howard Hughes Medical Institute and Abramson Family Cancer Research Institute, University of Pennsylvania Cancer Center, Philadelphia, Pennsylvania, USA
[3]Cancer Research UK, London Research Institute, London, United Kingdom

It has been proposed that bone marrow (BM) hematopoietic stem and progenitor cells are distributed along an oxygen (O_2) gradient, where stem cells reside in the most hypoxic areas and proliferating progenitors are found in O_2-rich areas. However, the effects of hypoxia on human hematopoietic stem cells (HSCs) have not been characterized. Our objective was to evaluate the functional and molecular responses of human BM progenitors and stem cells to hypoxic conditions. BM lineage-negative (Lin^-) $CD34^+CD38^-$ cells were cultured in serum-free medium under 1.5% O_2 (hypoxia) or 20% O_2 (normoxia) for 4 days. Using limiting dilution analysis, we demonstrate that the absolute number of SCID-repopulating cells (SRCs) increased by 5.8-fold in hypoxic cultures compared with normoxia, and by 4.2-fold compared with freshly isolated $Lin^-CD34^+CD38^-$ cells. The observed increase in BM-repopulating activity was associated with a preferential expansion of $Lin^-CD34^+CD38^-$ cells. We also demonstrate that, in response to hypoxia, hypoxia-inducible factor-1α protein was stabilized, surface expression of angiogenic receptors was upregulated, and VEGF secretion increased in BM Lin^-CD34^+ cultures. The use of low O_2 levels to enhance the survival and/or self-renewal of human BM HSCs in vitro represents an important advance and could have valuable clinical implications.

J. Clin. Invest. 112:126–135 (2003). doi:10.1172/JCI200317669.

Introduction

The effects of hypoxia on human hematopoietic progenitors and stem cells remain poorly understood. It has only recently been reported that, in normal volunteers, the partial pressure of oxygen (O_2) and O_2 saturation in human bone marrow (BM) are lower than in peripheral blood (1). The presence of hypoxic areas in the BM is determined by the architecture of medullary sinuses and the pattern of arterial blood flow in the marrow (2). It has been proposed that hematopoietic stem cells (HSCs) and progenitors are distributed along an O_2 gradient, with stem cells residing in the most hypoxic areas and proliferating progenitors in O_2-rich areas (3). The data in support of this model were generated using "closed systems" in which O_2 consumption by cells results in a progressive decrease in O_2 level below the initial 1%. In these conditions, Cipolleschi et al. (3) demonstrated that the ability of HSCs to repopulate the BM of lethally irradiated mice and give rise to myeloid colonies was better preserved in hypoxia (≤1.5% O_2 for 5 days) than in normoxic conditions. In contrast, committed progenitors (GM-CFU) could not be preserved under the same low O_2 levels. When an O_2 regulation device and growth factor stimulation were used, Ivanovic et al. (4) reported a smaller expansion of mouse progenitors and a better maintenance of the number of myeloid progenitors in the recipients' BM after 8 days in hypoxia compared with normoxia. In addition, we have shown that the proliferation of embryonic hematopoietic progenitors is regulated by a hypoxia-mediated signaling pathway (5).

The available data on the effects of hypoxia on human hematopoietic cells is limited to the clonogenic activity of umbilical cord blood, mobilized peripheral blood, or BM progenitor cells. Cipolleschi et al. (6) reported an increase in burst-forming unit, erythroid (BFU-E) and a decrease in GM-CFU colonies when cord blood $CD34^+$ cells were cultured in a 14-day clonogenic assay under severe hypoxia (<1% O_2). An increase in both BFU-E and GM-CFU activity in BM $CD34^+$ cells cultured in 1.5% O_2 for 6 hours has also been reported (7). In contrast, the culture of mobilized peripheral blood $CD34^+$ cells under severe hypoxia (<1% O_2) for 7

Received for publication December 19, 2002, and accepted in revised form April 22, 2003.

Address correspondence to: Guénahel H. Danet, Division of Hematology-Oncology, University of Pennsylvania School of Medicine, 723 Biomedical Research Building-II/III, 421 Curie Boulevard, Philadelphia, Pennsylvania 19104-6160, USA.
Phone: (215) 573-3705; Fax: (215) 573-7049;
E-mail: gdanet@mail.med.upenn.edu.
Dominique A. Bonnet and M. Celeste Simon contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: bone marrow (BM); hematopoietic stem cell (HSC); burst-forming unit, erythroid (BFU-E); colony-forming cell (CFC); SCID-repopulating cell (SRC); bone marrow lineage-negative (Lin^-); long-term colony-initiating cell (LTC-IC); hypoxia-inducible factor-1α (HIF-1α); aryl hydrocarbon receptor nuclear translocator protein (ARNT); phycoerythrin (PE); stem cell factor (SCF); VEGF receptor 2 (VEGFR2).

days resulted in a decrease in the total number of colony-forming cells (CFCs) (8). Although these in vitro assays provide clues on the effect of hypoxia on the clonogenic activity of human progenitors, they may not reflect its effects on human HSCs. It is necessary to use a xenotransplantation model, such as NOD/SCID mice (9), to evaluate the functional response of human BM-repopulating cells to culture under low O_2 levels. Human HSCs capable of extensive proliferation and multilineage repopulation of the BM of NOD/SCID mice are defined as SCID-repopulating cells (SRCs) (10–12). SRCs are highly enriched in the BM lineage-negative (Lin^-) $CD34^+CD38^-$ fraction (10–12) and present at a higher frequency in umbilical cord blood than in adult BM (13, 14). We and others (15, 16–24) have examined various culture conditions for their ability to maintain or enhance SRC activity in vitro. From these studies, it has been shown that the ex vivo expansion of CFCs and long-term colony-initiating cells (LTC-ICs) is not necessarily associated with an increase in SRCs. Furthermore, limiting dilution analysis is required to quantitatively compare the effect of culture conditions on SRC activity (12, 17–20, 24).

The cellular mechanisms by which human hematopoietic progenitors and stem cells respond to hypoxia have not been characterized. However, the response to hypoxia has been investigated in a variety of other cell types and models (see refs. 25–29 for reviews). The hypoxia-inducible factor-1α (HIF-1α) protein, which is rapidly degraded under normoxic conditions, becomes stabilized (30) under low O_2 levels (<5%) and forms a dimer with aryl hydrocarbon receptor nuclear translocator protein (ARNT). In contrast to HIF-1α, ARNT levels are not regulated by hypoxia. The heterodimer HIF-1α-ARNT is a transcriptional activator of genes encoding for a wide variety of genes including erythropoietin, VEGF, glucose transporters, and glycolytic enzymes (27, 31). HIF-2α (32–34) and HIF-3α (35) are closely related to HIF-1α but have a more restricted pattern of expression and partially overlapping functions (27).

The objectives of this study were to evaluate the functional and cellular responses of human BM hematopoietic progenitors and stem cells to hypoxia. We used normal adult BM as a source of human HSCs because these cells reside in a hypoxic environment in vivo (1) and therefore may represent a more relevant model than other sources of human HSCs such as cord blood or mobilized peripheral blood. In this study, we demonstrate quantitatively that adult BM SRCs can be expanded in vitro under hypoxic conditions. This increase in BM-repopulating activity was associated with the preferential expansion of $Lin^-CD34^+CD38^-$ cells, an upregulation of angiogenic receptors, and an increase in VEGF production. Our results suggest that hypoxia could play a critical role in regulating the self-renewal of human BM HSCs and that the increase in VEGF secretions induced by hypoxia could be involved in the maintenance of HSCs in vitro.

Methods

BM cell isolation. BM aspirates (n = 137) were obtained from healthy volunteers in accordance with the guidelines of the University of Pennsylvania Institutional Review Board for Human Subjects. At least three BM samples from three different donors were pooled for the isolation of Lin^- cells. Light-density mononuclear cells were isolated using Ficoll-Paque Plus (Amersham Biosciences Corp., Piscataway, New Jersey, USA), erythrocytes were lysed using ammonium chloride, and Lin^- cells were isolated using immunomagnetic beads (StemCell Technologies Inc., Vancouver, British Columbia, Canada). Briefly, cells were incubated with a mixture of lineage-specific antibodies (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, CD41, and glycophorin A) followed by incubation with a secondary antibody conjugated to metal colloid. Cells were then eluted through a magnetized column to deplete the suspension from cells expressing lineage markers. Lin^- cells were then stained with anti-human CD34-phycoerythrin (CD34-PE), anti-human CD38-allophycocyanine (both from Becton, Dickinson and Co., San Jose, California, USA), and propidium iodide (Molecular Probes Inc., Eugene, Oregon, USA) and sorted on a MoFlo cell sorter (Cytomation Inc., Fort Collins, Colorado, USA).

Liquid cultures. BM Lin^-CD34^+ or $Lin^-CD34^+CD38^-$ cells (10^6/ml) were cultured in serum-free medium consisting of StemSpan SFEM medium (StemCell Technologies Inc.) supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml), stem cell factor (SCF) (300 ng/ml), Flt-3 ligand (300 ng/ml), G-CSF (50 ng/ml), 1% HEPES, gentamicin, and LDL (10 μg/ml). Cells were cultured for 4–9 days at 37°C in 5% CO_2-humidified incubators in normoxic (20% O_2) or hypoxic (1.5% O_2) conditions. Hypoxic cultures were performed in a two-gas incubator (Jouan Inc., Winchester, Virginia, USA) equipped with an O_2 probe to regulate N_2 levels. In some experiments, normoxic cultures of BM Lin^-CD34^+ cells were supplemented with human VEGF (100 ng/ml), angiopoietin-1 (100 ng/ml), or angiopoietin-2 (100 ng/ml), all obtained from R&D Systems Inc. (Minneapolis, Minnesota, USA).

Phenotypic analysis and cell division tracking. Lin^-CD34^+ cells were harvested after 4 days of culture in hypoxic (1.5% O_2) or normoxic conditions. Cell number and viability were evaluated using trypan blue exclusion. Cells were incubated with anti-human CD34 and the following antibodies: CD31, CD38, CD117 (c-kit), and HLA-DR (Becton, Dickinson and Co.), CD90 from Beckman Coulter (Miami, Florida, USA), CD133 from Miltenyi Biotec (Auburn, California, USA), CD135 and CD162 from Immunotech Inc. (Westbrook, Maine, USA), CXCR4, Tie-1, Tie-2, Flt-1, and VEGF receptor 2 (VEGFR2, also known as Kdr, Flk-1) from R&D Systems Inc., and vWF from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). In some experiments, freshly isolated Lin^-CD34^+ cells were labeled with CFSE (Molecular Probes Inc., Eugene, Oregon, USA) as previously described (15). Cells were cultured

for 4 days in hypoxic (1.5% O_2) or normoxic conditions and stained for CD34-allophycocyanin and either the appropriate isotype control or one of the following anti-human antibodies: CD38, CD90, CD117, CD133, CD135, or HLA-DR. Cells were then analyzed on a FACSCalibur flow cytometer (Becton, Dickinson and Co.).

Cell cycle analysis. Lin^-CD34^+ cells cultured for 4 days in hypoxic (1.5% O_2) or normoxic conditions were harvested and cell cycle analysis was performed as previously described (36). Briefly, cells were incubated with CD34-PE, then fixed with a 0.4% formaldehyde-buffered solution, washed, and permeabilized with a 0.2% Triton X-100 solution on ice. After two washes in PBS plus 2% FBS, cells were labeled with Ki-67-FITC (Becton, Dickinson and Co.). Finally, each sample was washed twice and resuspended in a 10 μM solution of DAPI (Molecular Probes Inc.) in PBS plus 2% FBS. Cells were analyzed using a Becton-Dickinson LSR flow cytometer equipped with a 325-nm helium-cadmium UV laser and a 488-nm argon-ion laser.

CFC and LTC-IC assays. Human CFCs were assayed in semisolid methylcellulose medium in standard conditions (12). LTC-IC cultures were established on preformed M210B4 stroma layers using human myeloid long-term culture medium (MyeloCult 5100; StemCell Technologies Inc.) according to described methods (37, 38). Limiting dilutions (at least three replicates/dilution) were performed in 96-well plates in which 500–2,000 $Lin^-CD34^+CD38^-$ cells per well were plated. After 5 weeks in coculture with stroma cells, the content of each well was transferred in methylcellulose medium to reveal CFC activity.

Transplantation and analysis of NOD/SCID mice. Eight-week-old sublethally irradiated (275 cGy at 240 cGy/min) NOD/LtSz-scid (NOD/SCID) mice were transplanted with fresh or cultured BM $Lin^-CD34^+CD38^-$ cells by lateral tail vein injection according to a standard protocol (12). Cultured $Lin^-CD34^+CD38^-$ cells were not resorted for this phenotype prior to transplantation. $Lin^-CD34^+CD38^-$ cells cultured for 0, 4, 6, and 9 days in hypoxic or normoxic conditions were injected into mice at doses ranging from 300 to 40,000 cells per mouse (at least three mice per cell dose per experiment). $Lin^-CD34^+CD38^-$ cells were coinjected with 0.5 million to 1 million accessory cells consisting of irradiated (1,500 cGy) BM or cord blood mononuclear or lineage-positive cells as previously described (39). Mice were sacrificed 8–10 weeks after transplant and BM from the femurs, tibiae, and iliac crests of each mouse were harvested. To prepare mouse BM cells for flow cytometry, BM cells were incubated with a 6% ammonium chloride solution, then washed and incubated with anti-human CD45-FITC, CD33-PE, (Beckman Coulter), and CD19-allophycocyanin (Becton, Dickinson, and Co.), and propidium iodide. For each group of mice analyzed, an aliquot of cells was also stained with matching isotype controls. For each sample, 100,000 scatter- and live-gated cells were acquired to determine engraftment. Transplanted mice showing at least 0.1% human $CD45^+$ cells and both human myeloid ($CD33^+$) and lymphoid ($CD19^+$) cells were considered engrafted, as shown in Figure 3d.

RT-PCR. RNA was isolated from freshly purified $Lin^-CD34^+CD38^-$ or $Lin^-CD34^-CD38^-$ cells using the RNAqueous isolation kit (Ambion Inc., Austin, Texas, USA). RNA extracted from K562 cells was used as a positive control for the PCR reactions. Standard reverse transcription was done using SuperScript II reverse transcriptase (Invitrogen Corp., San Diego, California, USA). The primers used were HIF-1α: 5′-AAGTCTCGAGATGCAGCCAGA and 5′-AGTTAGTTCAAA CTGACTTAATCC; HIF-2α: 5′-GCCCCTGCTGTCCTGCCTCATCA and 5′-ATCCGTCTGGGTACTGCATTGGTCCTT; and ARNT: 5′-AGGAATAGTGGCCTAGCCCCT and 5′-ATTGTTGTAGCTGTTGCTCTG. PCR was done using the Advantage-GC cDNA PCR kit (Clontech Laboratories Inc., Palo Alto, California, USA). The amplification cycles were: 94°C for 4 minutes followed by 35 cycles of 94°C for 10 minutes + 58°C for 30 seconds + 72°C for 2 minutes, and a final extension step at 72°C for 10 minutes was included before samples were cooled at 4°C.

Western blot analysis. Equal numbers of Lin^-CD34^+ cells were cultured overnight in serum-free conditions (10^6/ml) at 1.5% O_2 in a hypoxic workstation (Invivo$_2$; Ruskinn Technology, Leeds, United Kingdom) or in normoxic conditions. Cells were directly lysed in 1× SDS loading buffer and boiled at 95°C for 10 minutes. The mouse anti–HIF-1α antibody used was from Becton, Dickinson and Co. (catalog no. H72320) and the rabbit anti-ARNT antibody was from Novus Biologicals Inc. (catalog no. NB 100-110; Littleton, Colorado, USA).

Human VEGF ELISA and lactate assay. Conditioned media from overnight cultures (n = 5) of Lin^-CD34^+ cells were harvested and human VEGF and erythropoietin concentrations were determined by ELISA (R&D Systems Inc.) according to the manufacturer's procedure. Lactate concentrations in the conditioned media were also determined after overnight culture in hypoxic (1.5% O_2) and normoxic conditions using a colorimetric assay (Sigma-Aldrich, St. Louis, Missouri, USA) based on the enzymatic conversion of lactate to pyruvate and H_2O_2 by lactate oxidase.

Statistics. Data are presented as mean ± SD or mean ± SEM. Statistical differences were evaluated using the Student t test. In the limiting dilution analysis used to determine SRC frequencies, mice with at least 0.1% human cells were considered engrafted. The data from limiting dilution experiments were analyzed using the single-hit Poisson model, and SRC frequencies were determined using the maximum likelihood estimator as previously shown (12, 13, 16–20, 24). We used χ^2 analysis to verify the internal consistency of our data and validate the use of Poisson statistics.

Results

Preferential expansion of $Lin^-CD34^+CD38^-$ cells in hypoxia. $Lin^-CD34^+$and $Lin^-CD34^+CD38^-$ cells were cultured in serum-free conditions for 4 days under normoxia (20% O_2) or hypoxia (1.5% O_2). We used this level of O_2 based

on previous reports indicating that mouse BM-repopulating cells can be maintained when cultured under 1% O_2 (3, 4). As shown in Figure 1a, the level of expansion of Lin$^-$CD34$^+$ cells was decreased in hypoxia compared with normoxia. In contrast, the expansion of Lin$^-$CD34$^+$CD38$^-$ cells, a subpopulation enriched in primitive progenitors and stem cells, was greater in hypoxic conditions than in normoxia (2.4-fold vs. 1.5-fold, respectively). The differential effect of hypoxia on subsets of Lin$^-$CD34$^+$ cells was further investigated using CFSE to track the division history of each primitive subset (CD90$^+$, HLA-DR$^-$, CXCR4$^+$, CD117^{low}, CD133$^+$, CD135$^+$) during the 4-day culture (Figure 1b). Overall, CFSE profiles indicated that all examined subsets of Lin$^-$CD34$^+$ cells cultured in hypoxia divided at a slower rate than in normoxia. In particular, HLA-DR$^-$ cells divided only once or twice during the 4-day culture period. Despite this general slow proliferative rate, a small fraction (4–18%) of the cells in each subset was associated with a lower CFSE fluorescence, indicative of a rapid rate of division. These results show that a small subset of primitive BM CD34$^+$ cells can rapidly proliferate under low O_2 levels while most CD34$^+$ cells have a decreased rate of division. This suggests that O_2 levels can differentially regulate the proliferation of primitive subsets of human BM cells.

Hypoxia regulates the cell cycle of Lin$^-$CD34$^+$ cells. We evaluated the effect of hypoxia on the cell cycle of Lin$^-$CD34$^+$ cells after 4 days of culture in hypoxic or normoxic conditions. Before culture, less than 1% of sorted Lin$^-$CD34$^+$ cells was found in the S or G_2/M fractions (data not shown). After 4 days under hypoxia, 34.2% ± 3.5% of CD34$^+$ cells was found in S + G_2/M phases compared with 46.3% ± 3.4% in normoxia (Table 1). A similar trend was observed for Lin$^-$CD34$^-$ cells (data not shown). These results indicate that the slower rate of expansion of Lin$^-$CD34$^+$ cells under hypoxia is at least partially due to slower cell cycling and fewer cell divisions (as shown by DAPI and CFSE, respectively). We also examined the effect of hypoxia on the transition from G_0 to G_1 phase using Ki-67 to distinguish cells in G_0 phase (Ki-67$^-$) from those in G_1 phase (Ki-67$^+$). Table 1 shows that, in normoxic conditions, the G_0/G_1 ratio (% cells in G_0 to % cells in G_1) was 87 ± 12, indicating that most cells found in the G_0 + G_1 peak (as defined by DAPI staining) were resting. In hypoxic conditions, the G_0/G_1 ratio was only 13 ± 3 due to a 6.7-fold increase in cells in G_1 phase and fewer cells in G_0 phase. These results suggest that hypoxia preferentially promotes the transition of Lin$^-$CD34$^+$ cells from G_0 to G_1 and/or induces a cell cycle arrest in G_1 phase.

Effects of hypoxia on CFCs and LTC-ICs. We evaluated the clonogenic activity of Lin$^-$CD34$^+$ cells after 4 days of culture in hypoxic or normoxic conditions (Figure 2). After 4 days of culture, the total number of CFCs was increased by twofold compared with freshly isolated Lin$^-$CD34$^+$ cells (Figure 2a). The level of O_2 had no significant effect on lineage-committed progenitors, with the exception of granulocytic colonies (G-CFU), which decreased in number under hypoxic conditions. We also examined the effect of hypoxia on more primitive progenitors. LTC-ICs were equally expanded (1.4-fold) after 4 days of culture in hypoxia or normoxia (Figure 2b). In normoxia, only LTC-IC granulocyte-macrophage colonies significantly increased while, in hypoxia, both granulocyte-macrophage and erythroid colonies

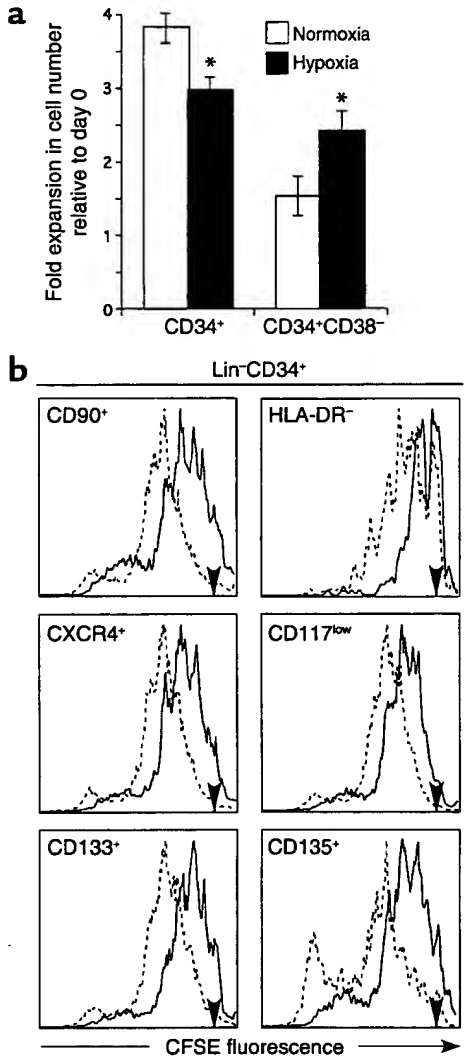


Figure 1
Expansion and division history of BM cells after 4 days in culture in hypoxia or normoxia. Lin$^-$CD34$^+$ and Lin$^-$CD34$^+$CD38$^-$ cells were cultured in serum-free conditions for 4 days in the presence of IL-3, IL-6, SCF, Flt-3 ligand, and G-CSF. (**a**) Expansion. The fold increase in cell number relative to the initial cell number plated (day 0) is represented for each subpopulation cultured in normoxia (white bars) or hypoxia (black bars). *$P < 0.05$. (**b**) Division history of primitive subpopulations of Lin$^-$CD34$^+$ cells. Freshly isolated Lin$^-$CD34$^+$ cells were labeled with CFSE, cultured for 4 days in hypoxia (1.5% O_2) or normoxia, and analyzed for CFSE fluorescence intensity and expression of markers associated with primitive progenitors and stem cells. Histograms (representative of three separate experiments) of CFSE fluorescence in various Lin$^-$CD34$^+$ cell subsets are shown after 4 days of culture in hypoxia (solid lines) or normoxia (dashed lines). Day 0 CFSE fluorescence intensity is indicated by an arrow on each histogram.

Table 1
Cell cycle analysis of BM Lin$^-$CD34$^+$ cells after 4 days of culture in hypoxia or normoxia

	Lin$^-$CD34$^+$	
	Hypoxia	Normoxia
% $G_0 + G_1$	65.8 ± 3.1	53.7 ± 4.0
G_0/G_1 ratio	13 ± 3	87 ± 12
% S	25.8 ± 2.3	34.2 ± 2.9
% G_2 + M	8.4 ± 1.2	12.1 ± 0.5

BM Lin$^-$CD34$^+$ cells were sorted at day 0 and analyzed by flow cytometry after 4 days in culture under hypoxia (1.5% O_2) or normoxia. Cells were stained with CD34, Ki-67, and DAPI. Values represent mean ± SD of three experiments.

increased. This increase in BFU-E was the only hypoxia-specific effect on primitive progenitors we observed. These results indicate that a 4-day culture in hypoxia has a very limited effect on both committed and primitive progenitors.

SCID-repopulating activity in cells cultured under hypoxic conditions. First, we evaluated the effect of hypoxia on the SCID-repopulating ability of BM Lin$^-$CD34$^+$CD38$^-$ cells cultured for 4, 6, and 9 days in the presence of IL-3, IL-6, SCF, Flt-3 ligand, and G-CSF. In these conditions, SRCs could be maintained for only 4 days in culture, regardless of O_2 levels. No human cells were detectable in the BM of mice (n = 16) transplanted with cells cultured for 6 or 9 days. These results indicate that in our conditions, hypoxia did not delay the loss of BM-repopulating activity observed after 4 days in normoxic cultures.

We used a limiting dilution analysis to quantitatively evaluate the effect of hypoxia on SCID-repopulating cells after 4 days. For this purpose, increasing numbers (ranging from 300 to 20,000) of fresh BM Lin$^-$CD34$^+$CD38$^-$ cells or cells cultured under hypoxia or normoxia for 4 days were transplanted at increasing doses into NOD/SCID mice (Figure 3). For each experimental condition (day 0, day 4 hypoxia, and day 4 normoxia), we estimated the number of Lin$^-$CD34$^+$CD38$^-$ cells required to statistically inject one SRC per mouse (12, 13, 20). Table 2 shows the frequencies of engrafted mice for each cell dose used in each experimental condition. We estimated that the frequency of SRCs in freshly purified Lin$^-$CD34$^+$CD38$^-$ cells (Table 2) was 1 in 1,009. After 4 days of culture, we determined that the frequency of SRCs in hypoxic conditions (1 in 577 Lin$^-$CD34$^+$CD38$^-$ cells) was significantly higher (P = 0.0017) than in normoxic cultures (1 SRC in 2,108 Lin$^-$CD34$^+$CD38$^-$ cells). Consequently, we observed a 3.6-fold increase in SRC frequency in hypoxic cultures compared with normoxia. In contrast, the apparent increase in SRC frequency between freshly isolated Lin$^-$CD34$^+$CD38$^-$ cells (1 SRC in 1,009 cells) and day 4 hypoxic cultures (1 in 577 cells) was not significant (P = 0.098). However, taking into account the expansion of Lin$^-$CD34$^+$CD38$^-$ CFCs after 4 days (Figure 1a), we estimate that in hypoxia, the absolute number of SRCs increased by 5.8-fold compared with the number in normoxia, and by 4.2-fold compared with freshly isolated Lin$^-$CD34$^+$CD38$^-$ cells. In contrast, in normoxic conditions, the absolute number of SRCs was just maintained in comparison with freshly isolated Lin$^-$CD34$^+$CD38$^-$ cells. In this study, we did not formally assess the effect of hypoxia on the self-renewal of human HSCs since no secondary transplants were performed. However, our results clearly demonstrate that low O_2 levels can substantially increase the number of BM-repopulating cells compared with normoxic conditions.

Molecular response of Lin$^-$CD34$^+$CD38$^-$ cells to hypoxia. We examined whether human BM progenitors and stem cells express the critical components of the cellular response to hypoxia, such as HIF-1α and ARNT. Figure 4a shows that both HIF-1α and ARNT are constitutively expressed in purified Lin$^-$CD34$^+$CD38$^-$ cells. HIF-2α expression could not be detected in Lin$^-$CD34$^+$CD38$^-$ cells but was present at very low levels in Lin$^-$CD34$^-$CD38$^-$ cells, a subpopulation enriched in CD34$^-$ SRCs (data not shown). Since HIF-α subunits are regulated at the protein level,

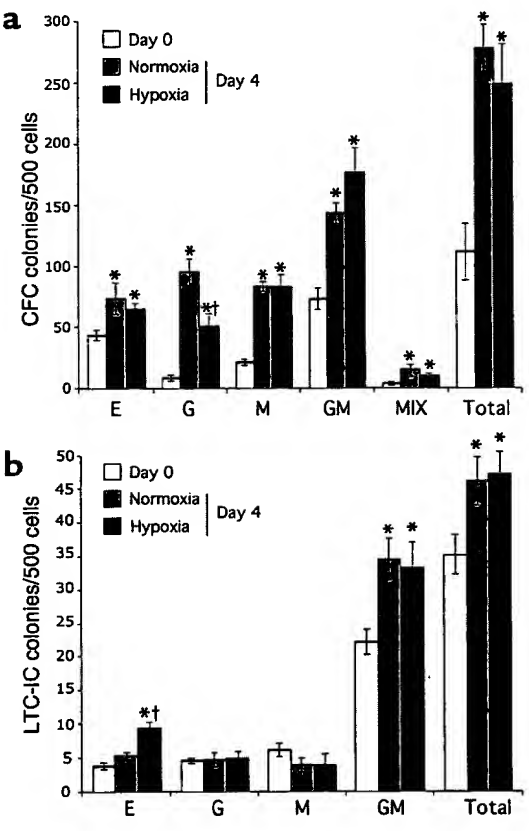


Figure 2
Effect of hypoxia on BM progenitor activity. The CFC (**a**) and LTC-IC (**b**) activity of BM Lin$^-$CD34$^+$CD38$^-$ cells was evaluated before (day 0; white bars) and after culture for 4 days under normoxic (gray bars) or hypoxic (1.5% O_2; black bars) conditions. Data shown represent the number (mean ± SD) of BFU-E (E), granulocyte (G), monocyte (M), granulocyte-monocyte (GM), mixed (MIX, i.e., GM colonies with erythroid cells), and total number of colonies from three separate experiments. The paired Student t test was performed to compare day 0 with day 4 cells (*P < 0.05) and to compare hypoxic to normoxic conditions after 4 days of culture (†P < 0.05).

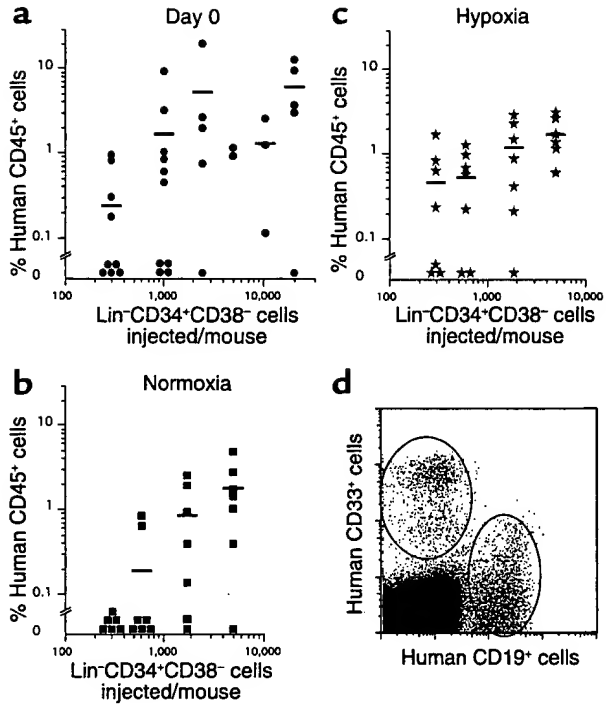


Figure 3
Quantitative analysis of the effect of hypoxia on SRC frequency. The frequency of SRCs in freshly isolated Lin$^-$CD34$^+$CD38$^-$ cells (**a**, day 0) or cells cultured for 4 days under normoxic (**b**) or hypoxic (1.5% O_2) (**c**) conditions was determined by limiting dilution analysis. For each NOD/SCID mouse (n = 88), the level of human engraftment (percentage of CD45$^+$ cells) is represented as a function of the number of Lin$^-$CD34$^+$CD38$^-$ cells transplanted. (**d**) Representative flow cytometry dot plot showing the presence of both human myeloid (CD33$^+$) and lymphoid (CD19$^+$) cells in NOD/SCID BM 6–8 weeks after transplantation.

we evaluated the effect of hypoxia and normoxia on HIF-1α protein. Figure 4b shows that HIF-1α protein can be detected in cells cultured in hypoxia but not under normal O_2 levels. In contrast, ARNT protein levels were unaffected by O_2 levels. These results demonstrate that HIF can mediate the hypoxic response of human BM progenitors and stem cells.

Hypoxia regulates expression of angiogenic factors in Lin$^-$CD34$^+$ cells. Angiogenic factors and receptors such as VEGF and Flt-1 are regulated by O_2 levels (27). VEGF has been implicated in the regulation of HSC survival (40) and recruitment (41). To investigate whether the increase in SRC activity observed under hypoxia was associated with changes in the expression of angiogenic molecules, Lin$^-$CD34$^+$ cells cultured for 4 days at 1.5% O_2 or at normoxia were analyzed by flow cytometry for cell surface expression of VEGF receptor 1 (VEGFR1, also known as Flt-1), VEGFR2, Tie-1, Tie-2, and vWF. Levels of VEGF cell surface receptors (VEGFR1 and VEGFR2) increased slightly, whereas angiopoietin receptors (Tie-1 and Tie-2) were more clearly upregulated in cells cultured in hypoxic conditions (Figure 5a). In contrast, c-kit and Flt-3 ligand were unchanged, and vWF and MHC class II (HLA-DR) expression was downregulated under the same conditions (Figure 5a). It is unlikely that the upregulation of VEGFR1, VEGFR2, Tie-1, and Tie-2 was due to a nonspecific response to low O_2 levels since other surface molecules were either unchanged or downregulated. These results suggest that human progenitors and stem cells can specifically enhance their responsiveness to angiogenic factors under hypoxic conditions.

Hypoxia-regulated genes include *VEGF* (42–44), erythropoietin (45), and glycolytic enzymes (46, 47) such as lactate dehydrogenase (48). To determine whether these HIF targets are upregulated in BM progenitors and stem cells, we cultured Lin$^-$CD34$^+$ cells for 18 hours under normoxic or hypoxic conditions and assayed VEGF, erythropoietin, and lactate concentrations in conditioned medium. Figure 5b shows that both VEGF and lactate secretions were increased by more than twofold, demonstrating that hypoxia upregulates VEGF production and glycolytic enzyme activity in human BM progenitors and stem cells. In contrast, erythropoietin was undetectable in the medium after 18 hours of culture regardless of O_2 levels, suggesting that HIF targets may be differentially regulated in BM cells. Our results indicate that the hypoxic response of human progenitors and HSCs is characterized by a rapid increase in VEGF secretion and glycolytic activity. This rapid increase in VEGF secretion may play a critical role in the survival and expansion of human BM-repopulating cells under hypoxia.

To determine whether angiogenic cytokines play a role in the upregulation of angiogenic receptors observed under hypoxic conditions, Lin$^-$CD34$^+$ cells were cultured in normoxia in the presence of VEGF, angiopoietin-1, or angiopoietin-2 for 4 days. Interestingly, VEGFR2 surface expression was enhanced to levels comparable to those observed under hypoxia when cells were cultured in the presence of either VEGF,

Table 2
Frequency of NOD/SCID engraftment observed with increasing doses of fresh or cultured Lin$^-$CD34$^+$CD38$^-$ cells after 4 days of culture in hypoxia or normoxia

	No. of NOD/SCID mice engrafted/transplanted		
	Day 0	Day 4	
No. injected cells		Normoxia	Hypoxia
300	4/9 (44%)	0/6 (0%)	4/7 (57%)
600	ND	2/7 (28%)	5/7 (71%)
1,000	6/10 (60%)	ND	ND
1,800	ND	5/7 (71%)	6/7 (85%)
2,500	4/5 (80%)	ND	ND
≥5,000	9/10 (90%)	6/7 (85%)	6/6 (100%)
SRC frequency	1 in 1,009	1 in 2,108[A]	1 in 577[B]

NOD/SCID mice (n = 88) were injected with increasing doses of BM Lin$^-$CD34$^+$CD38$^-$ cells and analyzed 8–10 weeks after transplantation. Mice with BM containing at least 0.1% human myeloid (CD33$^+$) and lymphoid (CD19$^+$) cells were considered engrafted. [A]Significantly different from day 0 (P = 0.04). [B]Significantly different from day 4 normoxia (P = 0.0017).

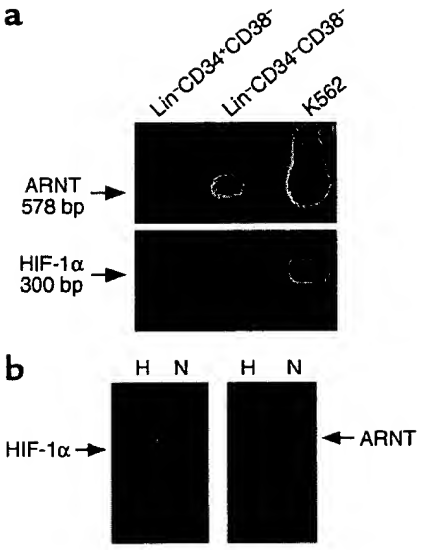


Figure 4
Expression of HIF in BM progenitors and stem cells. (**a**) RT-PCR analysis of ARNT and HIF-1α performed on RNA extracted from BM Lin$^-$CD34$^+$CD38$^-$, Lin$^-$CD34$^-$CD38$^-$, and K562 cells (as a positive control). (**b**) Western blot analysis of cell extracts from BM Lin$^-$CD34$^+$ cells cultured overnight under hypoxic (H) or normoxic (N) conditions using antibodies against ARNT and HIF-1α. All gels are representative of at least three experiments.

angiopoietin-1, or angiopoietin-2 (Figure 5c). In contrast, these cytokines had no effect on the expression or Flt-1, Tie-1, or Tie-2 (data not shown).

Discussion

In this report, we demonstrate for the first time that SRCs from human BM can be expanded in vitro under hypoxic conditions. We used BM Lin$^-$CD34$^+$CD38$^-$ cells, a population highly enriched in SRCs (12), and limiting dilution analysis to quantitatively evaluate the effect of hypoxia on SRCs. First, we determined that the frequency of SRCs in fresh adult BM is 1 in 1,009 Lin$^-$CD34$^+$CD38$^-$ cells. This result demonstrates that the frequency of SRCs in adult BM is only half of the SRC frequency that was reported previously for cord blood (1 in 617 Lin$^-$CD34$^+$CD38$^-$ cells) (12). This is consistent with other reports indicating that the frequency of SRC is higher in cord blood than in BM or mobilized peripheral blood (13, 14). To evaluate the effect of hypoxia on BM SRCs in vitro, we used culture conditions (IL-3, IL-6, SCF, Flt-3 ligand, and G-CSF in serum-free medium) capable of expanding SRCs in cord blood Lin$^-$CD34$^+$CD38$^-$ cells (16). Interestingly, after 4 days in the same culture conditions, SRCs in BM Lin$^-$CD34$^+$CD38$^-$ cells could not be expanded. The lack of SRC expansion after 4 days in culture under normoxic conditions suggests that human adult BM HSCs have different culture/cytokine requirements than cord blood HSCs. In contrast to normoxic conditions, we observed a significant increase in SRCs after 4 days in culture under 1.5% O_2. Not only were SRCs increased relative to normoxia, but we also observed a 4.2-fold expansion of the number of SRCs in hypoxic cultures compared with freshly isolated BM Lin$^-$CD34$^+$CD38$^-$ cells. These results demonstrate that low levels of O_2 can greatly enhance the survival and/or self-renewal of human BM-repopulating cells in vitro. This finding is consistent with a previous report indicating that mouse BM-repopulating cells were better maintained in culture under severe hypoxia than were more committed progenitors (3). We could no longer detect SRC activity after 6 or 9 days in culture under 1.5% or 3% (data not shown) or 20% O_2. This finding indicates that, in the present culture conditions, which may not be optimal for BM stem cells, the positive effect of hypoxia on SRCs is short-lived.

The effect of hypoxia on clonogenic (CFC) and LTC-IC activity was limited to a reduction of granulocytic progenitors (G-CFU) and an increased number of LTC-ICs giving rise to BFU-E. The limited response of BM progenitors to hypoxia compared with the significant increase in SRCs observed in the same conditions demonstrates that the activity of human BM stem cells and progenitors is regulated differently by O_2.

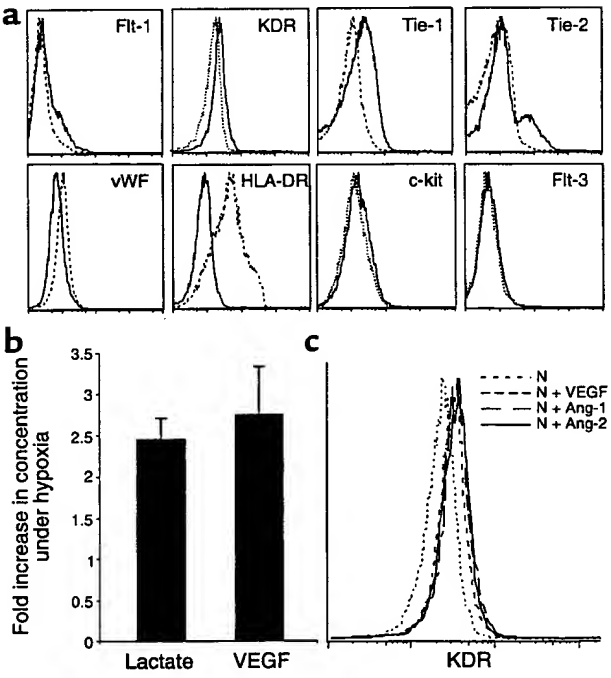


Figure 5
Hypoxia regulates the expression of HIF target genes in BM progenitors and stem cells. (**a**) Representative histograms of cell surface expression of angiogenic and hematopoietic cytokine receptors in BM Lin$^-$CD34$^+$ cells after 4 days in culture under normoxic (dashed lines) or hypoxic (1.5% O_2, solid lines) conditions. (**b**) The conditioned medium from BM Lin$^-$CD34$^+$ cells cultured overnight in normoxia or hypoxia (1.5% O_2) was assayed for lactate and human VEGF. Bars represent the fold increase in lactate and VEGF concentrations measured in hypoxic cultures relative to normoxic conditions. Error bars show SD. (**c**) Effect of VEGF, angiopoietin-1 (Ang-1), and angiopoietin-2 (Ang-2) on cell surface expression of VEGFR2 in Lin$^-$CD34$^+$ cells after 4 days in culture under normoxic conditions (N).

To further characterize the hypoxic response, we evaluated the effects of low O_2 tension on the proliferation and cell cycle distribution of human BM progenitors and stem cells in vitro. The expansion of Lin^-CD34^+ cells was slightly reduced at 1.5% O_2 compared with normoxia, indicating that most BM progenitors can survive and even proliferate under hypoxia. Interestingly, the expansion of purified $Lin^-CD34^+CD38^-$ cells was greater in hypoxic than in normoxic conditions. This positive effect of hypoxia on $Lin^-CD34^+CD38^-$ cells demonstrates that primitive progenitors and stem cells are particularly well adapted to proliferating in a low-O_2 environment.

To further evaluate the effect of hypoxia on the proliferation of BM cells, we used CFSE to track the division history of primitive subsets ($CD90^+$, $CXCR4^+$, $CD117^{low}$, $CD135^+$, and $HLA\text{-}DR^-$) of BM Lin^-CD34^+ cells cultured under hypoxia and normoxia. We show that under hypoxic conditions, the overall proliferative rates of Lin^-CD34^+ cells and subsets was slower in hypoxic conditions, resulting in fewer cells after 4 days in culture compared with normoxia. Hypoxia increased the percentage of the cells in G_0 or G_1 phase, while fewer cells were in found in S or G_2/M. Furthermore, hypoxic conditions increased the proportion of cells in G_1 phase relative to resting (G_0) cells, resulting in a dramatically decreased G_0 to G_1 ratio. This observation suggests that low O_2 levels can either promote the transition from a resting state to G_1 phase and/or inhibit the transition from G_1 to S phase in BM progenitors and stem cells. It has been shown that hypoxia can induce G_1 arrest in tumor (49, 50) and endothelial cells (51). Gardner et al. (52) reported that, in fibroblasts, severe hypoxia can inhibit the G_1/S transition through regulation of p27 expression. Wilpshaar et al. (53) found no difference in SCID-repopulating ability between uncultured cord blood $CD34^+$ cells in G_0 phase and those in G_1 phase. However, in cultured mobilized peripheral blood $CD34^+$ cells, the level of human chimerism in NOD/SCID BM was lower in mice transplanted with cells in G_1 phase compared with G_0 (54). The expansion of SRCs we observed after 4 days in hypoxia and the increased number of cells in G_1 phase suggests that SRCs may be found in both G_0 and G_1 phases under low oxygen levels.

In this study, we also investigated the cellular mechanisms involved in the hypoxic response of BM hematopoietic progenitor and stem cells. We found that HIF-1α and ARNT are constitutively expressed in $Lin^-CD34^+CD38^-$ cells. However, HIF-2α transcripts could not be detected in $Lin^-CD34^+CD38^-$ cells. Our Western blot analysis of Lin^-CD34^+ cells shows that HIF-1α protein is present under hypoxic conditions and is undetectable in normoxia. In contrast, ARNT protein is detectable regardless of O_2 levels. This is consistent with previous reports showing that, in normoxic conditions, HIF-1α is modified by a prolyl hydroxylase, an O_2-dependent enzyme (55, 56). The modified form of HIF-1α can interact with the von Hippel-Lindau tumor suppressor protein (VHL). This interaction induces the ubiquitination of HIF-1α and its rapid degradation (57–60). Under hypoxic conditions, the hydroxylation no longer occurs, and HIF-1α remains stable and can upregulate expression of its target genes.

Among the genes induced by hypoxia (25–29), angiogenic factors and receptors appear to play a critical role in the regulation of HSC survival and self-renewal. In this report we show an increase in VEGF secretion and an upregulation of the cell surface expression of angiogenic receptors (Tie-2 and to a lesser extent VEGFR2 and Tie-1) under hypoxia. The hypoxia-induced increase in VEGF expression has been well characterized in other models (42–44). Recently, Gerber et al. (40) reported that VEGF regulates the survival of mouse HSCs through an internal autocrine loop. It has also been shown that human BM-repopulating cells express VEGF receptors (61, 62). It is thus tempting to speculate that a similar mechanism may be involved in regulating the survival of SRCs. Tie-2, the receptor for angiopoietin-1, has also been reported to be regulated by O_2 levels (63) and can be found on HSCs (64). Thus, the combined effect of increased VEGF and enhanced responsiveness to angiogenic growth factors may play a role in the expansion of human BM-repopulating cells we observed. Future studies will be needed to further investigate the role of VEGF in the expansion of SRCs under hypoxia.

In conclusion, we show that the use of low O_2 levels in vitro not only improves SRC survival compared with normoxia but also expands the number of human BM repopulating cells by 4.2-fold compared with freshly isolated $Lin^-CD34^+CD38^-$ cells. The present report also establishes that hypoxia increases both VEGF secretions and responsiveness to angiogenic factors in BM progenitors and stem cells. In addition, we show that stem cells and progenitors respond differently to hypoxic conditions. These findings suggest that the balance between the survival, self-renewal, and differentiation of human BM HSCs might be tightly regulated by O_2 levels. The beneficial effect of hypoxia on human BM-repopulating cells may be mediated by angiogenic factors such as VEGF. Finally, the use of low O_2 tension could represent an important advance in the ex vivo expansion of human HSCs and may have important translational and clinical implications.

Acknowledgments

We are grateful to Jennifer Sinibaldi, Matthew Lessie, Amy Cleck, and Martin Carroll from the Stem Cell and Leukemia Core at the University of Pennsylvania Cancer Center for providing the large number of normal BM samples necessary for this study. The authors also thank William Demuth at the Abramson Family Cancer Research Center (University of Pennsylvania) for cell sorting, Tammi Coleman and Derrick Dow for their help with the NOD/SCID mouse colony, and Diana

Ramirez-Bergeron, Andy Arsham, Brian Keith, and other members of the Simon Laboratory for their valuable input. M.C. Simon is an Investigator of the Howard Hughes Medical Institute.

1. Harrison, J.S., Rameshwar, P., Chang, V., and Bandari, P. 2002. Oxygen saturation in the bone marrow of healthy volunteers. *Blood.* **99**:394.
2. Lichtman, M.A. 1981. The ultrastructure of the hemopoietic environment of the marrow: a review. *Exp. Hematol.* **9**:391–410.
3. Cipolleschi, M.G., Dello Sbarba, P., and Olivotto, M. 1993. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood.* **82**:2031–2037.
4. Ivanovic, Z., et al. 2000. Incubation of murine bone marrow cells in hypoxia ensures the maintenance of marrow-repopulating ability together with the expansion of committed progenitors. *Br. J. Haematol.* **108**:424–429.
5. Adelman, D.M., Maltepe, E., and Simon, M.C. 1999. Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. *Genes Dev.* **13**:2478–2483.
6. Cipolleschi, M.G., et al. 1997. Severe hypoxia enhances the formation of erythroid bursts from human cord blood cells and the maintenance of BFU-E in vitro. *Exp. Hematol.* **25**:1187–1194.
7. Quinlan, D.P., Jr., et al. 1998. Effect of hypoxia on the hematopoietic and immune modulator preprotachykinin-I. *Arch. Surg.* **133**:1328–1334.
8. Ivanovic, Z., Dello Sbarba, P., Trimoreau, F., Faucher, J.L., and Praloran, V. 2000. Primitive human HPCs are better maintained and expanded in vitro at 1 percent oxygen than at 20 percent. *Transfusion.* **40**:1482–1488.
9. Dick, J.E. 1996. Normal and leukemic human stem cells assayed in SCID mice. *Semin. Immunol.* **8**:197–206.
10. Larochelle, A., et al. 1996. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nat. Med.* **2**:1329–1337.
11. Pflumio, F., et al. 1996. Phenotype and function of human hematopoietic cells engrafting immune-deficient CB17-severe combined immunodeficiency mice and nonobese diabetic-severe combined immunodeficiency mice after transplantation of human cord blood mononuclear cells. *Blood.* **88**:3731–3740.
12. Bhatia, M., Wang, J.C.Y., Kapp, U., Bonnet, D., and Dick, J.E. 1997. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **94**:5320–5325.
13. Wang, J.C., Doedens, M., and Dick, J.E. 1997. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood.* **89**:3919–3924.
14. Holyoake, T.L., Nicolini, F.E., and Eaves, C.J. 1999. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. *Exp. Hematol.* **27**:1418–1427.
15. Danet, G.H., Lee, H.W., Luongo, J.L., Simon, M.C., and Bonnet, D.A. 2001. Dissociation between stem cell phenotype and NOD/SCID repopulating activity in human peripheral blood CD34(+) cells after ex vivo expansion. *Exp. Hematol.* **29**:1465–1473.
16. Bhatia, M., et al. 1997. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. *J. Exp. Med.* **186**:619–624.
17. Conneally, E., Cashman, J., Petzer, A., and Eaves, C. 1997. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. *Proc. Natl. Acad. Sci. U. S. A.* **94**:9836–9841.
18. Piacibello, W., et al. 1999. Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. *Blood.* **93**:3736–3749.
19. Bhatia, M., et al. 1999. Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *J. Exp. Med.* **189**:1139–1148.
20. Ueda, T., et al. 2000. Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J. Clin. Invest.* **105**:1013–1021.
21. Bryder, D., and Jacobsen, S.E. 2000. Interleukin-3 supports expansion of long-term multilineage repopulating activity after multiple stem cell divisions in vitro. *Blood.* **96**:1748–1755.
22. Kollet, O., et al. 2000. The plant lectin FRIL supports prolonged in vitro maintenance of quiescent human cord blood CD34(+)CD38(-/low)/SCID repopulating stem cells. *Exp. Hematol.* **28**:726–736.
23. Shih, C.C., et al. 2000. A secreted and LIF-mediated stromal cell-derived activity that promotes ex vivo expansion of human hematopoietic stem cells. *Blood.* **95**:1957–1966.
24. Chute, J.P., et al. 2002. Ex vivo culture with human brain endothelial cells increases the SCID-repopulating capacity of adult human bone marrow. *Blood.* **100**:4433–4439.
25. Harris, A.L. 2002. Hypoxia—a key regulatory factor in tumour growth. *Nat. Rev. Cancer.* **2**:38–47.
26. Kaelin, W.G., Jr. 2002. How oxygen makes its presence felt. *Genes Dev.* **16**:1441–1445.
27. Wenger, R.H. 2002. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *FASEB J.* **16**:1151–1162.
28. Semenza, G.L. 2001. HIF-1 and mechanisms of hypoxia sensing. *Curr. Opin. Cell Biol.* **13**:167–171.
29. Ramirez-Bergeron, D.L., and Simon, M.C. 2001. Hypoxia-inducible factor and the development of stem cells of the cardiovascular system. *Stem Cells.* **19**:279–286.
30. Jiang, B.H., Semenza, G.L., Bauer, C., and Marti, H.H. 1996. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. *Am. J. Physiol.* **271**:C1172–C1180.
31. Semenza, G.L. 1998. Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis. *J. Lab. Clin. Med.* **131**:207–214.
32. Tian, H., McKnight, S.L., and Russell, D.W. 1997. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* **11**:72–82.
33. Ema, M., et al. 1997. A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc. Natl. Acad. Sci. U. S. A.* **94**:4273–4278.
34. Flamme, I., et al. 1997. HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. *Mech. Dev.* **63**:51–60.
35. Gu, Y.Z., Moran, S.M., Hogenesch, J.B., Wartman, L., and Bradfield, C.A. 1998. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene Expr.* **7**:205–213.
36. Guzman, M.L., et al. 2001. Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells. *Blood.* **98**:2301–2307.
37. Hogge, D.E., Lansdorp, P.M., Reid, D., Gerhard, B., and Eaves, C.J. 1996. Enhanced detection, maintenance, and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human steel factor, interleukin-3, and granulocyte colony-stimulating factor. *Blood.* **88**:3765–3773.
38. Sutherland, H.J., Eaves, C.J., Lansdorp, P.M., Thacker, J.D., and Hogge, D.E. 1991. Differential regulation of primitive human hematopoietic cells in long-term cultures maintained on genetically engineered murine stromal cells. *Blood.* **78**:666–672.
39. Bonnet, D., Bhatia, M., Wang, J.C., Kapp, U., and Dick, J.E. 1999. Cytokine treatment or accessory cells are required to initiate engraftment of purified primitive human hematopoietic cells transplanted at limiting doses into NOD/SCID mice. *Bone Marrow Transplant.* **23**:203–209.
40. Gerber, H.P., et al. 2002. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. *Nature.* **417**:954–958.
41. Hattori, K., et al. 2001. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J. Exp. Med.* **193**:1005–1014.
42. Liu, Y., Cox, S.R., Morita, T., and Kourembanas, S. 1995. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer. *Circ. Res.* **77**:638–643.
43. Forsythe, J.A., et al. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* **16**:4604–4613.
44. Levy, A.P., Levy, N.S., Wegner, S., and Goldberg, M.A. 1995. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J. Biol. Chem.* **270**:13333–13340.
45. Wang, G.L., and Semenza, G.L. 1993. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. *J. Biol. Chem.* **268**:21513–21518.
46. Semenza, G.L., Roth, P.H., Fang, H.M., and Wang, G.L. 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Biol. Chem.* **269**:23757–23763.
47. Firth, J.D., Ebert, B.L., Pugh, C.W., and Ratcliffe, P.J. 1994. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3′ enhancer. *Proc. Natl. Acad. Sci. U. S. A.* **91**:6496–6500.
48. Firth, J.D., Ebert, B.L., and Ratcliffe, P.J. 1995. Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. *J. Biol. Chem.* **270**:21021–21027.
49. Graeber, T.G., et al. 1996. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature.* **379**:88–91.
50. Schmaltz, C., Hardenbergh, P.H., Wells, A., and Fisher, D.E. 1998. Reg-

ulation of proliferation-survival decisions during tumor cell hypoxia. *Mol. Cell. Biol.* **18**:2845–2854.
51. Iida, T., et al. 2002. Hypoxia-inducible factor-1alpha induces cell cycle arrest of endothelial cells. *Genes Cells.* **7**:143–149.
52. Gardner, L.B., et al. 2001. Hypoxia inhibits G1/S transition through regulation of p27 expression. *J. Biol. Chem.* **276**:7919–7926.
53. Wilpshaar, J., et al. 2000. Similar repopulating capacity of mitotically active and resting umbilical cord blood CD34(+) cells in NOD/SCID mice. *Blood.* **96**:2100–2107.
54. Gothot, A., van der Loo, J.C., Clapp, D.W., and Srour, E.F. 1998. Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood CD34(+) cells in non-obese diabetic/severe combined immune-deficient mice. *Blood.* **92**:2641–2649.
55. Jaakkola, P., et al. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science.* **292**:468–472.
56. Ivan, M., et al. 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science.* **292**:464–468.
57. Cockman, M.E., et al. 2000. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J. Biol. Chem.* **275**:25733–25741.
58. Ohh, M., et al. 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat. Cell Biol.* **2**:423–427.
59. Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. 2000. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. *EMBO J.* **19**:4298–4309.
60. Kamura, T., et al. 2000. Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc. Natl. Acad. Sci. U. S. A.* **97**:10430–10435.
61. Ziegler, B.L., et al. 1999. KDR receptor: a key marker defining hematopoietic stem cells. *Science.* **285**:1553–1558.
62. Hattori, K., et al. 2002. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat. Med.* **8**:841–849.
63. Oh, H., et al. 1999. Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J. Biol. Chem.* **274**:15732–15739.
64. Hashiyama, M., et al. 1996. Predominant expression of a receptor tyrosine kinase, TIE, in hematopoietic stem cells and B cells. *Blood.* **87**:93–101.

nature medicine

Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1

Daniel J Ceradini, Anita R Kulkarni, Matthew J Callaghan, Oren M Tepper, Nicholas Bastidas, Mark E Kleinman, Jennifer M Capla, Robert D Galiano, Jamie P Levine & Geoffrey C Gurtner

The trafficking of circulating stem and progenitor cells to areas of tissue damage is poorly understood. The chemokine stromal cell–derived factor-1 (SDF-1 or CXCL12) mediates homing of stem cells to bone marrow by binding to CXCR4 on circulating cells[1,2]. SDF-1 and CXCR4 are expressed in complementary patterns during embryonic organogenesis and guide primordial stem cells to sites of rapid vascular expansion[3]. However, the regulation of SDF-1 and its physiological role in peripheral tissue repair remain incompletely understood[4]. Here we show that SDF-1 gene expression is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1) in endothelial cells, resulting in selective *in vivo* expression of SDF-1 in ischemic tissue in direct proportion to reduced oxygen tension. HIF-1–induced SDF-1 expression increases the adhesion, migration and homing of circulating CXCR4-positive progenitor cells to ischemic tissue. Blockade of SDF-1 in ischemic tissue or CXCR4 on circulating cells prevents progenitor cell recruitment to sites of injury. Discrete regions of hypoxia in the bone marrow compartment also show increased SDF-1 expression and progenitor cell tropism. These data show that the recruitment of CXCR4-positive progenitor cells to regenerating tissues is mediated by hypoxic gradients via HIF-1–induced expression of SDF-1.

Tissue repair and regeneration after injury is thought to involve the selective recruitment of circulating or resident stem cell populations[4]. The importance of SDF-1 in stem and progenitor cell recruitment has been established with observations that selective expression in injured tissue correlates with adult stem cell recruitment and tissue regeneration[5,6]. However, the physiological mechanism underlying the localized expression of SDF-1 in injured tissue is unknown. Many factors produced during tissue injury could potentially regulate SDF-1 expression, including inflammatory mediators (interleukin-1 or tumor-necrosis factor-α), changes in the extracellular matrix and altered mechanical forces. Because hypoxia is a common theme in the protean pathways in which SDF-1 is essential[7], here we examined whether localized differences in SDF-1 expression and progenitor cell trafficking could be explained by local differences in oxygen tension.

We investigated the potential impact of reduced oxygen tension on SDF-1 regulation *in vivo* by using a model of soft-tissue ischemia in athymic nude mice[8] (Fig. 1a). Direct measurement of oxygen tension allowed us to define three discrete tissue segments (denoted A–C) with increasingly ischemic microenvironments (Fig. 1a and Supplementary Methods online). In this model, the nadir of tissue oxygenation occurs in the first 12 h and is followed by a progressive increase in oxygen tension over the course of 14 d (Fig. 1a) with a mild inflammatory response (Fig. 1b). We observed a marked increase in SDF-1 mRNA in ischemic tissue 6 h after surgery that was directly proportional to the reduction in tissue oxygen tension and resulted in a similar increase in SDF-1 protein expression (Fig. 1c).

Immunohistochemical analysis detected no SDF-1 protein in non-ischemic tissue (Fig. 1d,e). By contrast, SDF-1 was abundant in ischemic tissue in a vascular and perivascular distribution, both in the endothelial cells and lining the vascular lumen (Fig. 1d,e). The colocalization of CD31 and SDF-1 immunostaining suggested that endothelial cells were a source of SDF-1 expression in ischemic tissue (Fig. 1f), which was confirmed by *in situ* hybridization (Fig. 1g,h).

To study the molecular mechanism of hypoxia-induced SDF-1 expression, we examined human endothelial cells (HUVECs) *in vitro*. Oxygen levels in tissue culture were maintained at 1% (7.2 mmHg), which corresponded to the most ischemic tissue area in the mouse model (area C, Fig. 1a). Secreted SDF-1 was increased sevenfold in culture media after 6 h of hypoxia and reached a maximum ninefold increase by 12 h (Fig. 2a). Flow cytometry detected an increase in endothelial cell surface–bound SDF-1 (Fig. 2a), probably through SDF-1 binding to heparan sulfates on the endothelial surface[9]. Quantitative real-time RT-PCR showed a threefold increase in the number of SDF-1 mRNA transcripts after 3, 6 and 9 h of hypoxia (Fig. 2b). These findings suggest that hypoxia-specific transcriptional elements are a primary control mechanism for SDF-1 expression.

HIF-1 is the central mediator of the cellular response to hypoxia, regulating the expression of over 60 genes that affect cell survival and metabolism in adverse conditions[10]; however, HIF-1 has not been shown to regulate directly the expression of a member of the chemokine family, such as SDF-1. Analysis of the 5′ flanking region of the human *SDF1* gene identified, on the basis of previously published consensus

Laboratory of Microvascular Research and Vascular Tissue Engineering, Institute of Reconstructive Plastic Surgery, New York University School of Medicine, New York, New York 10016, USA. Correspondence should be addressed to G.C.G. (geoffrey.gurtner@med.nyu.edu).

Published online 4 July 2004; doi:10.1038/nm1075

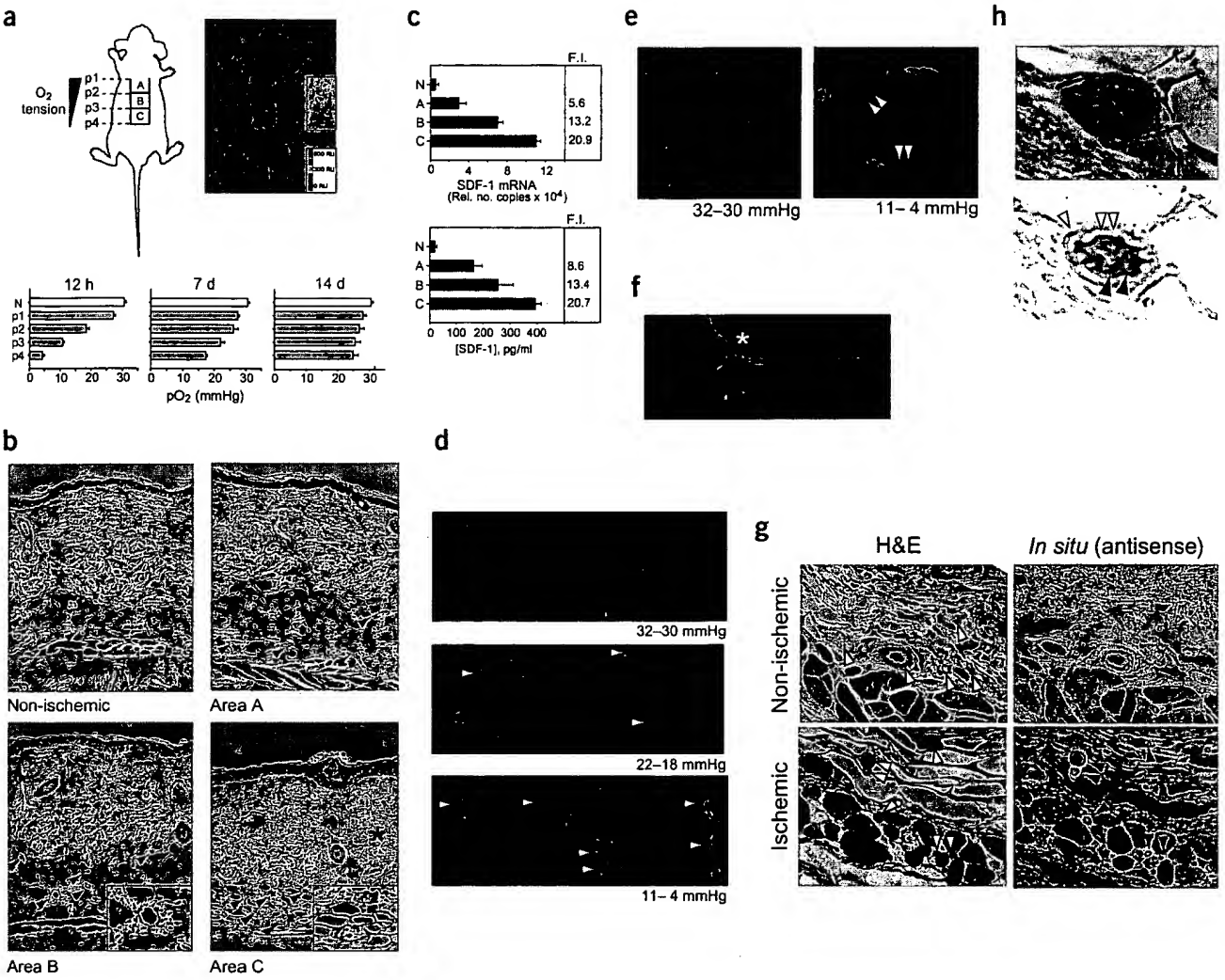


Figure 1 SDF-1 expression is directly proportional to reduced tissue oxygen tension *in vivo*. (**a**) Top, areas A–C in the mouse model show increasingly ischemic tissue, determined by direct oxygen tension measurements at points p1–p4 and laser Doppler at 12 h after surgery. Bottom, oxygen tension at p1–p4 and in non-ischemic tissue (N) after 12 h, 7 d and 14 d. (**b**) Hematoxylin and eosin (H&E) staining of tissue 14 d after surgery (original magnification, ×100). Mild perivascular infiltration of monocyte-like cells in more ischemic regions (areas B and C; inset, ×200) and a slight increase in dermal cellularity (area C; asterisk) accompany increased capillary density (arrowheads). (**c**) Top, quantitative real-time RT-PCR of total RNA collected from non-ischemic tissue (N) and areas A–C at 6 h after surgery. The mean fold induction (F.I.) over non-ischemic levels is indicated on the right. Bottom, SDF-1 protein expression at 12 h. (**d**) Expression of SDF-1 *in vivo*. Shown is SDF-1 (red, indicated by arrows; ×200) in non-ischemic tissue (top), area A (middle) and area C (bottom) at 12 h after surgery. Vessels are stained green by FITC-lectin perfusion. (**e**) Axial sectioned blood vessels (×400) from non-ischemic tissue (left) and area C (right); double-headed arrows indicate SDF-1 immunostaining (red). Green staining is FITC-lectin perfusion; blue is DAPI. (**f**) Colocalization (asterisk) of the endothelial marker CD31 (green) and SDF-1 (red; ×400). (**g**,**h**) *In situ* hybridization of SDF-1 mRNA. (**g**) H&E staining (open arrowheads indicate vessels; ×200) and *in situ* mRNA hybridization of serial sections indicating endothelial cell localization of SDF-1 mRNA (filled arrowheads indicate purple staining). (**h**) Expression of SDF-1 mRNA by ischemic blood vessel endothelium (double filled arrowheads; ×400), with little or no expression seen in smooth muscle (double open arrowheads), pericytes (open arrowhead) and surrounding stromal cells. Upper panel shows H&E staining with no immunostaining.

sequences (A/GCGTG), two potential HIF-1-binding sites termed HBS1 and HBS2 at nucleotides −1,238 and −783, respectively (Fig. 2c).

We cloned the 5′ 1.4-kilobase putative *SDF1* promoter sequence from human genomic DNA into a luciferase reporter vector to obtain pGL3b.*SDF1*.full (**Supplementary Methods** online). Transient transfection of this construct into a human microvascular endothelial cell line (HMEC-1) showed that *SDF1*-specific luciferase expression increased fourfold after exposure to either hypoxic conditions or 0.1 mM cobalt chloride, a known HIF-1 activator (Fig. 2c). Serial 5′ deletion analysis of the *SDF1* promoter indicated that removal of the putative HIF-1-binding sites abolished hypoxia and cobalt chloride–inducible gene expression, and site-directed mutagenesis of HBS1 in the full-length construct produced a similar effect (Fig. 2c). Insertion of these elements upstream of a minimal SV40 promoter confirmed that HBS1 is sufficient to confer hypoxia-specific gene expression (Fig. 2d).

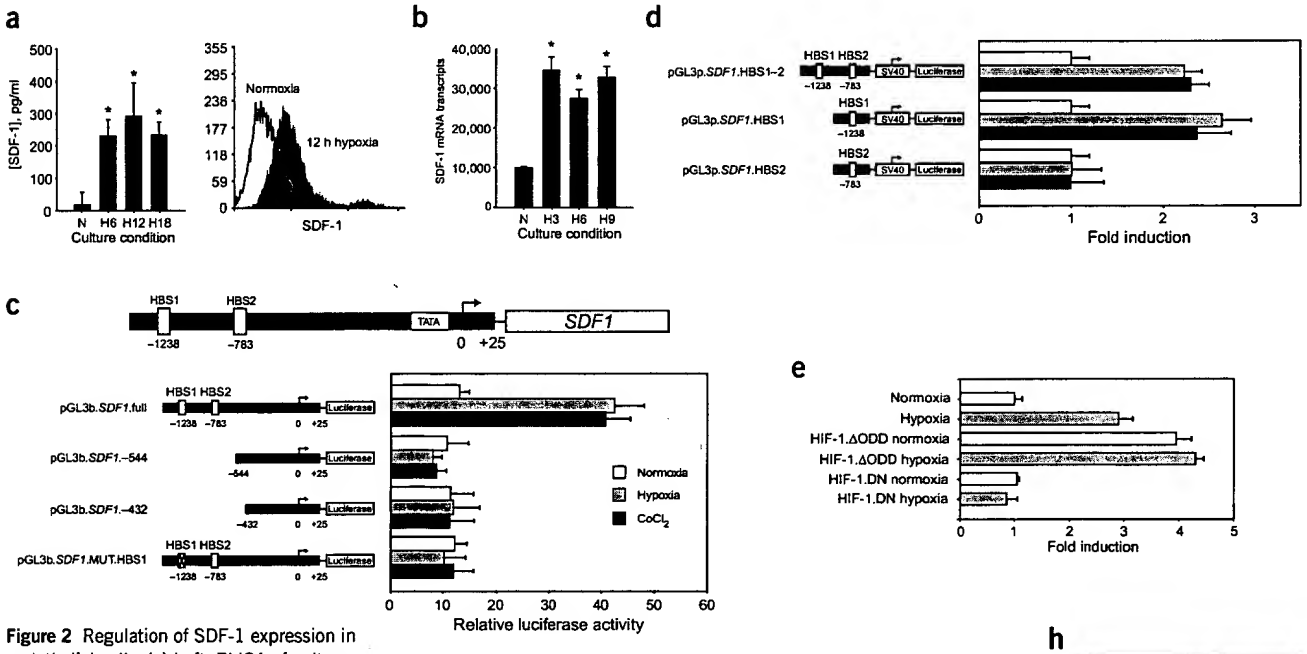
To verify the specificity of HIF-1 activation of SDF-1 transcription, we cotransfected HMECs with the full-length *SDF1* construct reporter and constructs encoding either a constitutively active, oxygen-independent HIF-1α mutant (HIF-1.ΔODD, which lacks the oxygen

dependent degradation domain)[11] or a constitutively expressed, HIF-1α dominant-negative variant (HIF-1.DN, which lacks the basic DNA-binding and carboxy-terminal transactivation domains)[12]. Coexpression of HIF-1.ΔODD and the reporter induced a fourfold transcriptional activation of the *SDF1* promoter in both normoxic and hypoxic conditions, whereas coexpression of HIF-1.DN abolished hypoxia-responsive expression (Fig. 2e). Selective gene silencing of HIF-1α with specific short interfering RNA (siRNA) also abolished HIF-1α expression in hypoxic conditions and blocked hypoxia-specific SDF-1 expression as compared with control siRNA (Fig. 2f and **Supplementary Methods** online).

Chromatin immunoprecipitation (ChIP) assays with HIF-1α antibodies and subsequent quantitative real-time PCR showed that seven fold more HIF-1α was directly bound to the *SDF1* promoter in hypoxic conditions (Fig. 2g). *In vivo*, HIF-1α and SDF-1 colocalized in roughly 76% of ischemic endothelial cells, mainly those lining small caliber arterioles, venules and capillaries (Fig. 2h). These data show that SDF-1 expression is transcriptionally activated by oxygen-dependent stabilization of HIF-1α and represent the first evidence, to our knowledge, of direct chemokine regulation by HIF-1.

We examined the functional significance of HIF-1-induced SDF-1 expression in endothelial cells by using adhesion assays *in vitro*. Previous studies have shown that chemokines enhance integrin-mediated adhesion of circulating leukocytes to endothelial monolayers[2,13]. CXCR4 is known to be highly expressed on numerous putative stem and progenitor cells, including hematopoietic, skeletal and smooth muscle, neural and endothelial precursors[1,6,14,15]. We used endothelial progenitor cells (EPCs) in these studies because ischemia-induced neovascularization is essential for tissue regeneration[16], and these cells are the most widely studied type of vascular progenitor cell[6,17,18].

We found that EPCs express high levels of CXCR4, as well as CD31 and KDR/FLK-1, with more than 94% expressing both CD31 and CXCR4 (Fig. 3a and **Supplementary Methods** online). HUVEC monolayers preconditioned in hypoxic conditions for 6 h were found to adhere a greater number of $CXCR4^+$ EPCs than were monolayers cultivated in normal oxygen tension (69.0%±0.52 versus 46.4%±2.3, $P < 0.001$), an effect that could be abolished by antibody blockade of SDF-1–CXCR4 interactions (Fig. 3b). Silencing of HIF-1α expression in endothelial cells with siRNA also abolished the hypoxia-specific increase in adhesion (Fig. 3c). Increased adhesion of $CXCR4^+$ EPCs to



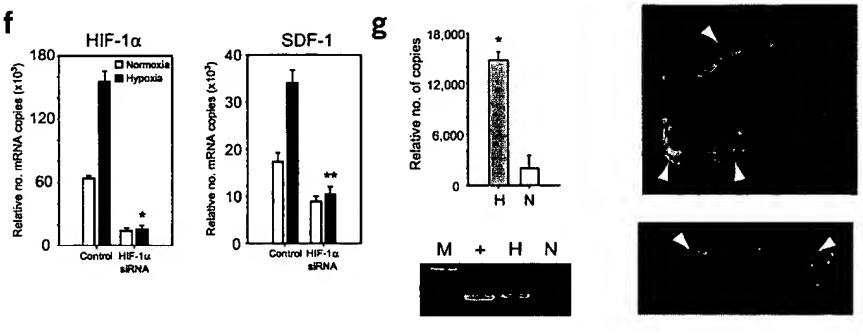


Figure 2 Regulation of SDF-1 expression in endothelial cells. (**a**) Left, ELISA of culture supernatants (HUVEC) after 6–18 h of hypoxic (H6–H18) or normoxic (N) conditions (*$P <$ 0.005). Right, surface-bound *SDF-1* during hypoxic (shaded) and normoxic (open) culture. (**b**) SDF-1 mRNA transcripts in normoxic (N) and hypoxic (H) conditions (*$P < 0.005$). (**c**) Top, human *SDF1* locus with HBS sequences and transcriptional start site (0). Bottom, luciferase activity of the pGL3b.SDF1.full, serial 5′ deletions, and HBS1 mutant constructs. (**d**) Luciferase activity of constructs with HIF-1-binding sites inserted 5′ to the minimal SV40 promoter. (**e**) Luciferase activity of the pGL3b.*SDF1*.full construct co-transfected with the HIF-1.ΔODD and HIF-1.DN constructs under normoxic or hypoxic conditions. (**f**) Effect of HIF-1α gene silencing on HIF-1α and SDF-1 mRNA expression (*$P < 0.005$, **$P < 0.01$). (**g**) ChIP analysis of *SDF1*-specific genomic sequences from endothelial cells cultivated in normoxic (N) or hypoxic (H) conditions using HIF-1α monoclonal antibodies (M, marker; +, plasmid control). (**h**) *In vivo* colocalization (arrowheads) of HIF-1α (green) and SDF-1 (red) in postcapillary venules of ischemic tissue (original magnification, ×400).

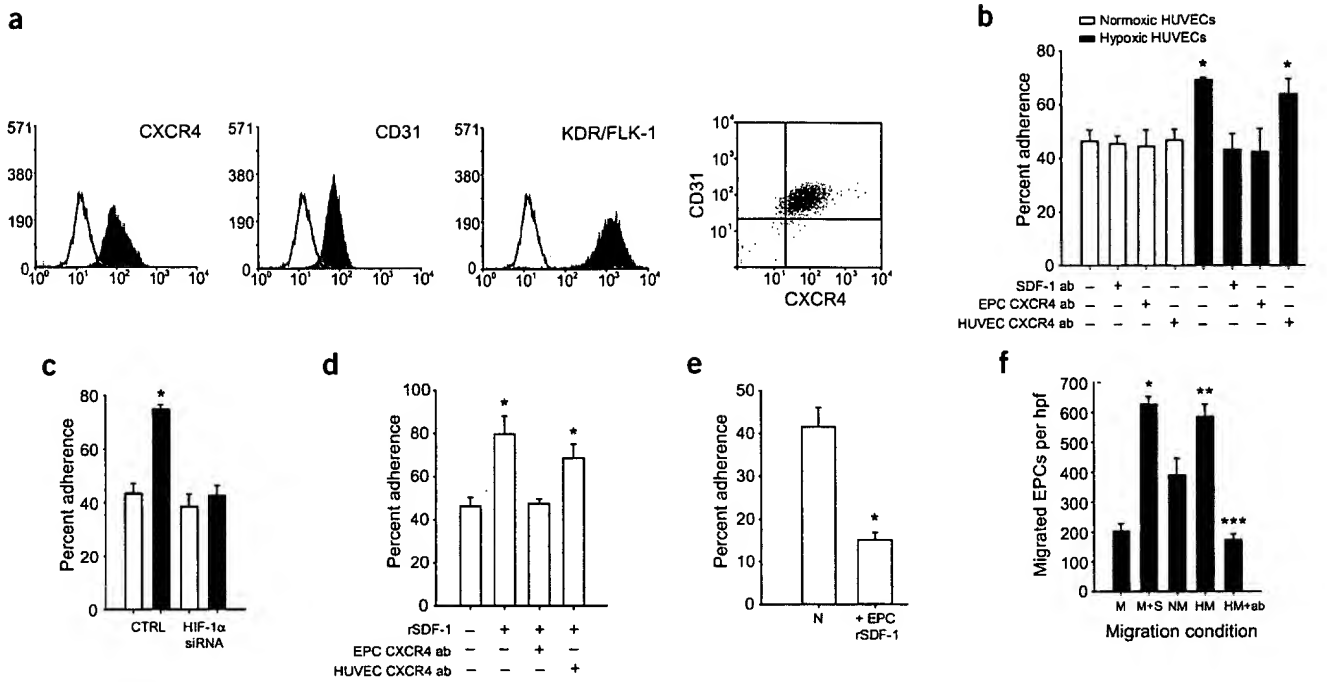


Figure 3 Functional interactions between endothelial cells and progenitor cells mediated by HIF-1-induced SDF-1 expression. (**a**) High levels of CXCR4, KDR/FLK-1 and CD31 expression in EPCs, with more than 94% of EPCs expressing both CD31 and CXCR4. (**b**) Hypoxia-conditioned HUVEC monolayers adhere to significantly more EPCs, an effect that is specific to interactions between SDF-1 and CXCR4 (*$P < 0.001$); ab, antibody. (**c**) Silencing of HIF-1α in HUVECs abolishes hypoxia-specific adhesion (*$P < 0.005$). (**d**) Normoxic HUVEC monolayers adhere more EPCs after being coated with SDF-1 (*$P < 0.001$); rSDF-1, recombinant SDF-1. (**e**) Preincubation of EPCs with SDF-1 before the assay reduces baseline adhesion (*$P < 0.005$). (**f**) EPC migration increases in response to media with recombinant SDF-1 (M+S) versus media alone (M; *$P < 0.001$). As compared with normoxic media (NM), hypoxia-conditioned media from HUVECs (HM) stimulates an increase in migrating EPCs (* *$P < 0.001$), which can be blocked by preincubating EPCs with neutralizing CXCR4 antibody (HM + ab; * * *$P < 0.001$).

normoxic HUVEC monolayers could be reproduced by precoating monolayers for 20 min with recombinant SDF-1 (Fig. 3d).

Notably, preincubation of CXCR4$^+$ EPCs with soluble SDF-1 200 ng/ml for 20 min before the assay reduced adhesion below baseline levels (41.6%±4.5 versus 15.1%±1.74, $P < 0.005$), presumably because of a reduction in available cell-surface receptors owing to rapid CXCR4 internalization[19] (Fig. 3e). This observation suggests that circulating SDF-1 may desensitize stem and progenitor cells in the bone marrow, thereby increasing their likelihood of mobilization[20], whereas SDF-1 immobilized on and around ischemic blood vessels facilitates tissue-specific adhesion and localization.

SDF-1 is also known to mediate the mobilization and migration of bone marrow–derived stem and progenitor cells *in vivo*[20,21]. We found that conditioned media from HUVECs cultivated in hypoxic conditions enhanced EPC migration as compared with media from HUVECs grown in normal oxygen tension (585±21 cells per high-power field (hpf) versus 389±18.25cells/hpf, $P < 0.001$, Fig. 3f). This increase in migration could be blocked by preincubating EPCs with neutralizing antibodies to CXCR4 (585±21 cells/hpf versus 175±9.8 cells/hpf, $P < 0.001$), whereas isotype control antibodies had no effect on migration (data not shown). Recombinant SDF-1 also induced robust migration in a dose-dependent manner as compared with controls, with a maximal response at 200 ng/ml (625±18 cells/hpf versus 203±10 cells/hpf, $P < 0.001$). These experiments show that HIF-1-induced expression of SDF-1 mediates functional interactions between mature endothelium and circulating progenitor cells.

We used the mouse ischemia model described above to examine both the influence of absolute tissue oxygen tension on circulating progenitor cell recruitment and whether HIF-1-induced SDF-1 expression is necessary for recruitment to occur. Previous studies have shown that EPCs localize to ischemic tissue and participate in tissue repair in animal models[6,16]. We found that the number of CXCR4$^+$ EPCs homing to (day 2 after surgery; data not shown) and engrafting in (day 14; Fig. 4a,b) ischemic tissue after intravascular administration was directly proportional to reduced tissue oxygen tension. The pattern of EPC engraftment was identical to the pattern of SDF-1 expression shown above. These cells rarely localized to vessels in non-ischemic tissue; by contrast, they were frequently found lining microvascular conduits in ischemic tissue (Fig. 4c).

To determine whether this engraftment was predominantly due to HIF-1-induced SDF-1 expression, we used specific antibody blockade of CXCR4 and SDF-1 to examine the contribution of this pathway to progenitor cell localization *in vivo*, because blockade of HIF-1 would have many nonspecific downstream effects (such as alterations in VEGF and iNOS expression), and both CXCR4- and SDF-1-null mice have an embryonic lethal phenotype[22,23]. Ischemia-specific homing and engraftment of CXCR4$^+$ EPCs was markedly reduced by preincubating cells with neutralizing CXCR4 antibody before administration despite the persistence of a hypoxic microenvironment ($n = 4$, Fig. 4d). In addition, intravenous administration of free neutralizing SDF-1 antibodies with progenitor cells reduced ischemia-specific engraftment to a similar degree ($n = 4$, Fig. 4d).

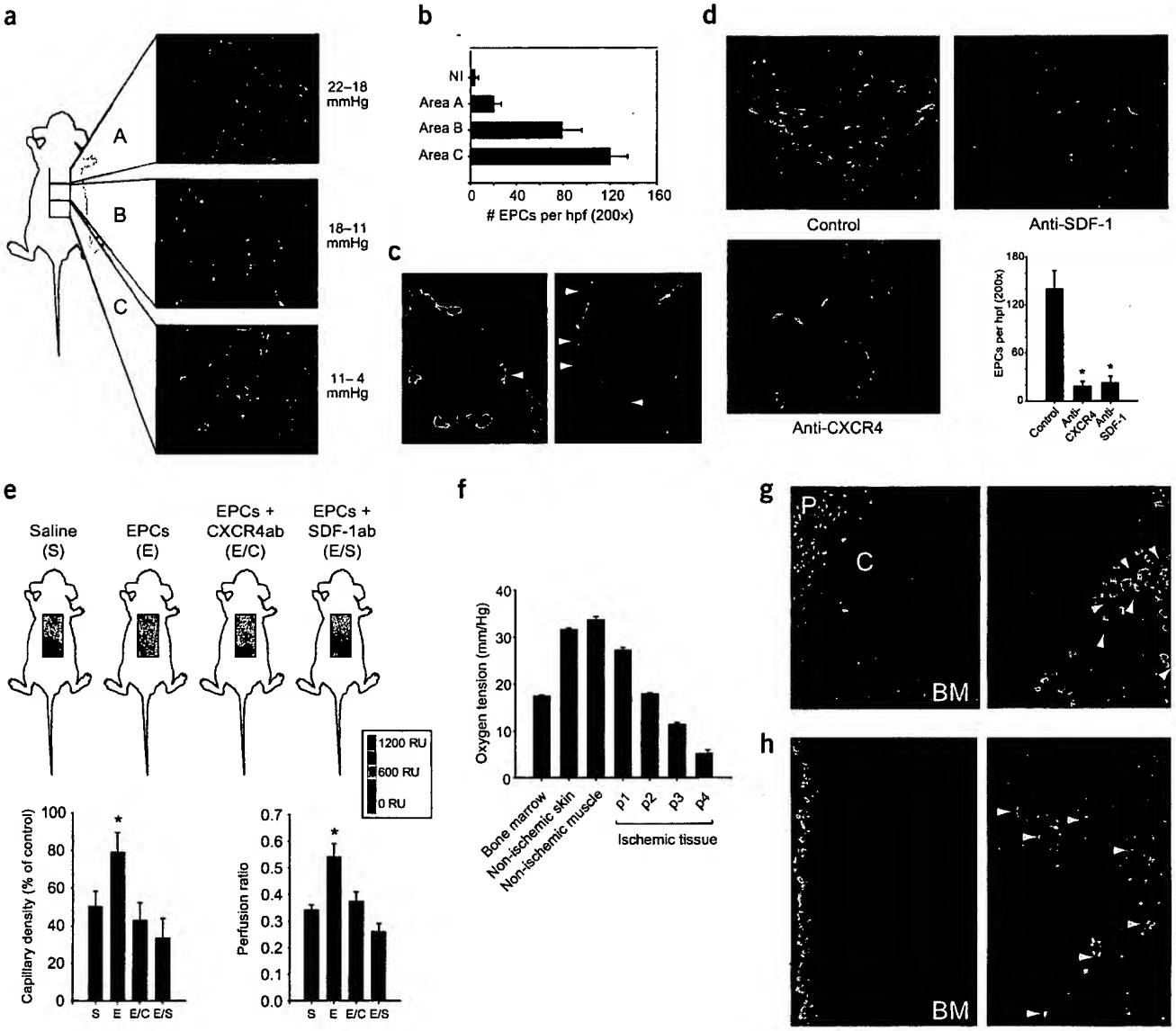


Figure 4 Dependence of EPC recruitment on HIF-1-induced expression of SDF-1. (**a**,**b**) DiI-labeled EPCs (red) engraft ischemic tissue in proportion to reduced oxygen tension (vessels stained with FITC-lectin; original magnification, ×200), as compared with non-ischemic tissue (NI). (**c**) EPCs (arrows) are rarely identified in non-ischemic tissue lining blood vessels (left), but frequently found in ischemic tissue lining functional microvascular channels (right; original magnification, ×400). (**d**) Blockade of CXCR4 of EPCs or coadministration of free neutralizing antibodies to SDF-1 significantly reduces the number of EPCs (red) that engraft ischemic tissue (*$P < 0.0001$, ×200). (**e**) Top, Doppler blood flow in ischemic tissue after 14 d in mice treated with saline (S), EPCs (E), EPCs preincubated with anti-CXCR4 (E/C), or EPCs coadministered with anti-SDF-1 (E/S). Bottom, relative blood flow (*$P < 0.01$) and capillary density (*$P < 0.001$) in area C of each group ($n = 4$). (**f**) Oxygen tension in the bone marrow, non-ischemic tissues and ischemic tissue in this mouse model (p1–p4). (**g**) Left, DAPI-stained bone marrow section, showing the bone marrow compartment (BM), cortical bone (C) and non-ischemic periosteal tissue (P). Right, same section showing colocalization of pimonidazole (green) and SDF-1 (red, indicated by arrows) immunostaining (×200). (**h**) Longitudinal DAPI-stained section of bone marrow (left), showing localization of EPCs (right; red, indicated by arrowheads) to SDF-1-rich regions (green) after intravascular administration (×200).

Functionally, EPC administration significantly improved ischemic tissue perfusion and capillary density after 14 d as compared with control mice, an effect that could be abrogated by CXCR4 or SDF-1 blockade ($n = 4$, Fig. 4e). Notably, blockade of host-derived SDF-1 resulted in greater impairments in perfusion and capillary density at 14 d, probably because it interfered with native mouse endothelial progenitor trafficking and neovascularization. Notably, administration of CXCR4$^+$ EPCs 7 d after ischemic surgery, when tissue oxygen tension has been restored, did not result in significant engraftment (data not shown). These results show that hypoxia-induced SDF-1 expression is crucially important in the selective homing and migration of CXCR4$^+$ progenitor cells to ischemic tissues, and suggests that induction of SDF-1 expression via HIF-1 can directly guide regenerative progenitor cells to areas of injury.

These data raise the question of whether ischemia is required for progenitor cell localization, maintenance and regeneration. If so, we

would expect that previously described stem and progenitor cell niches would be locally hypoxic. This idea has been suggested in a previous report indicating that bone marrow aspirates are hypoxic[24]. Direct examination of the bone marrow and uninjured tissues of mice showed that the oxygen tension in the bone marrow compartment *in situ* was consistently lower than in other tissues and, in fact, very similar to ischemic tissue in our model (Fig. 4f). Microscopic analysis showed that the bone marrow compartment contained discrete regions of hypoxia defined by pimonidazole localization that were associated with abundant SDF-1 immunostaining (Fig. 4g). Systemically administered EPCs specifically homed to (day 2) and engrafted (up to day 21) these regions regardless of the presence of a peripheral ischemic stimulus (Fig. 4h). These heterogeneous regions of hypoxia in the bone marrow microenvironment may explain the constitutive and regional expression of SDF-1 in the bone marrow and the CXCR4-dependent stem and progenitor cell tropism to the bone marrow[2].

Together with the finding that HIF-1 regulates CXCR4 (ref. 25), our data suggest that tissue hypoxia may be a fundamental mechanism governing stem and progenitor cell recruitment and retention. As such, transiently hypoxic microenvironments (such as injured tissue) may represent a conditional stem and progenitor cell niche, in which HIF-1 stabilization and activation of both the trafficking stimulus (SDF-1) and receptor (CXCR4) facilitate progenitor cell recruitment and retention in ischemic tissue requiring repair. This idea is supported by the dose-dependent relationship among absolute tissue oxygen tension, SDF-1 expression and progenitor cell recruitment. In addition, progenitor cells do not localize in tissue after normal oxygen tension has been restored and SDF-1 has returned to the low steady-state amounts observed in uninjured tissues, which implies that progenitor cell–mediated tissue regeneration may require a locally hypoxic milieu for success. Thus, manipulation of HIF-1 activity may be a useful means with which to augment the body's innate reparative capacity[26]. A reduction in HIF-1 activity, as occurs in aging[27], may also alter stem and progenitor cell trafficking and underlie the observed decline in regenerative capacity.

Similar to tissue injury, neoplastic states are often characterized by profound hypoxia. Studies have indicated that stem and progenitor cells and cancer cells share the CXCR4–SDF-1 axis for selective tissue homing[25,28]. This suggests that the tumor-associated microenvironment may continuously recruit circulating stem and progenitor cells, effectively 'hijacking' the body's capacity for tissue regeneration; in support of this, overexpression of HIF-1 is a negative prognostic indicator in many human cancers[10]. This implies that efforts to decrease tumor vascularity (such as anti-angiogenesis approaches) may be counterproductive because they increase tumor hypoxia, thereby potentially enhancing the recruitment of circulating stem and progenitor cells and enlisting host mechanisms for survival and growth.

METHODS

Mouse ischemia model and recruitment experiments. We used a model of ischemia in athymic nude mice (Jackson Laboratories) as described[8] in full accordance with the New York University Institutional Animal Care and Use Committee. In this model, a peninsular shaped incision was made that divided the epidermis, papillary and reticular dermis, subcutaneous connective tissue and skeletal muscle (panniculus carnosus) from the systemic circulation on all but a single side, generating a reproducible gradient of ischemia. After surgery, mice received an intracardiac injection of EPCs (5×10^5) labeled with dioctadecyl-tetramethylindo-carbocyanine perchlorate (DiI; Molecular Probes), or DiI-labeled EPCs preincubated with a control HLA class I antibody (PharMingen), preincubated with neutralizing CXCR4 antibody (clone 44716; R&D) or mixed with free HLA class I or neutralizing SDF-1 antibody (clone 79014.111; R&D). Free antibodies (without cells) were readministered via intraperitoneal injection 24 h after surgery[1]. Before being killed on day 2 or 14 after surgery, some mice were perfused with fluorescein isothiocyanate (FITC)-labeled *Lycopersicon esculentum* lectin (Vector) to stain the functional microvasculature. Collected tissue was snap frozen in liquid nitrogen, sectioned and mounted in 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) medium (Vector) and/or stained for immunofluorescence microscopy.

For EPC recruitment experiments, mice were killed on day 2 or 14 after surgery and the number of DiI-labeled EPCs from five nonconsecutive sections of each tissue area (A–C and non-ischemic) was quantified at ×200 magnification in three random fields by a investigator who was blinded to the samples. We determined functional capillary density on day 14 by *in situ* staining with FITC-lectin[29] as above. The endothelial phenotype of lectin-positive cells was confirmed by the colocalization of immunostaining for DiI, indicating human endothelial cells, and phycoerythrin (PE)-conjugated anti-CD31 (PharMingen), indicating mouse endothelial cells. For SDF-1, HIF-1α and CD31 detection (without EPC administration), 10-μm sections were incubated with FITC-conjugated anti-CD31, anti–mouse HIF-1α monoclonal antibody (Novus), and/or rabbit anti–mouse SDF-1 (e-Bioscience), and the unconjugated primary antibodies were detected by appropriate conjugated secondary antibodies (Alexa Fluor 594 or 488; Molecular Probes). We used the Hypoxyprobe-1 kit (pimonidazole hydrochloride; Chemicon) according to the manufacturer's protocol with the supplied monoclonal antibody and the Alexa Fluor 488–conjugated secondary antibody used for tissue detection.

***In situ* hybridization.** Frozen and fixed tissue sections (10 μm) were washed in PBS containing 0.2% RNase Block (InnoGenex), treated with proteinase K (20 μg/ml) for 10 min at 37 °C, and acetylated for 10 min at room temperature. Digoxigenin-labeled antisense RNA probes were generated from nucleotides 1,090–1,318 of the mouse SDF-1 complementary DNA sequence by a commercially available kit (Roche). Hybridization was done at 60 °C for 16 h with antisense RNA probe (200 ng/ml) in a commercially available hybridization buffer (InnoGenex). After post-hybridization washes, SDF-1 mRNA signal was detected with alkaline phosphatase–coupled anti-digoxigenin antibodies (diluted 1:1,000; Roche) overnight at 4 °C and developed with BM-Purple AP substrate containing 1 mM Levamisole. We used sense probes as controls.

Quantitative real-time RT-PCR. Total RNA was extracted from cultured cells or homogenized tissue with Tri-Reagent (Sigma) and purified by an RNAeasy kit (Qiagen). We used an RNA PCR Core kit (Applied Biosystems) to construct the template cDNA for real-time PCR (Cepheid Smartcycler; primers listed in Supplementary Methods online) using Platinum SYBR Green Supermix-UDG (Invitrogen). Relative quantification of PCR products was calculated after normalization to β-actin or glyceraldehyde-3-phosphate dehydrogenase. Results are representative of three independent experiments. Products were sequenced to confirm their identity.

SDF-1 ELISA. Enzyme-linked immunosorbent assay (ELISA) was done with a human or mouse SDF-1 Quantikine kit (R&D), used in accordance with the manufacturer's protocol. Protein extracted from tissue homogenates with the TPER reagent (Pierce) and cell culture supernatants were standardized for total protein content using the BCA Protein Assay kit (Pierce) before analysis. Results are representative of three independent experiments.

Cell culture. HUVECs (Clonetics) and HMEC-1 cells (CDC, Atlanta, GA) were cultivated in EGM-2 media (Clonetics). We collected human EPCs from healthy donors as described[8,16], after obtaining informed consent in accordance with the New York University Institutional Review Board. EPC identification and estimation of culture purity (90–95%) were determined by the percentage of cells that costained with FITC-labeled *Ulex europaeus* lectin I (UEA-1; Vector) and the uptake of DiI-conjugated acLDL (acetylated low-density lipoprotein), and confirmed by the coexpression of CD31 (refs. 8,30). Hypoxic culture conditions (1% O_2) were achieved in a custom-designed hypoxic incubator by a continuous infusion of a pre-analyzed gas mixture (95% N_2, 5% CO_2). Media was serum-starved in EBM-2 plus 0.5% fetal bovine serum (FBS) at least 8 h before hypoxic culture to minimize the effects of growth factors in the expansion media.

ChIP assays. HUVECs were grown to 90% confluence and exposed to normoxic or hypoxic conditions, and ChIP was done with a commercially available kit (Upstate), used in accordance with the manufacturer's protocol. We used antibodies to HIF-1α or mouse IgG (PharMingen) to immunoprecipitate DNA fragments, which we analyzed by real-time quantitative PCR with primers specific for the *SDF1* promoter (Supplementary Methods online), β-actin or glyceraldehyde-3-phosphate dehydrogenase. Products were sequenced to confirm their identity.

Adhesion assays. Confluent HUVEC monolayers were subjected to normoxic or hypoxic culture for 6 h. Human SDF-1αβ (100 ng/ml; Sigma) was added where indicated. SDF-1 antibody was added to the HUVEC monolayer after normoxic/hypoxic conditioning but before adding the EPCs. DiI-labeled EPCs (5×10^4) in EBM-2 plus 0.5 FBS were added to each preconditioned monolayer and pre-adhesion fluorescence was measured using a Cytofluor2320 (Millipore). After 3 h, nonadherent cells were washed away and post-adhesion fluorescence was measured. We calculated the percentage of adherent cells by the following formula: percentage of cells bound = (post-adhesion fluorescence–monolayer only)/(pre-adhesion fluorescence – monolayer only) × 100. Results are representative of three independent experiments.

Migration assays. Migration was studied by a modified trans-well assay. EPCs (5×10^4) were seeded onto Chemotx filters (5.7-mm, 8-μm pore; Neuro Probe) in EBM plus 0.5% FBS. Recombinant human SDF-1αβ (Sigma) and conditioned media (EBM plus 0.5% FBS) from HUVECs cultured in different oxygen tensions for 6 h were then added to the lower chamber. After the 6-h migration period, nonmigrating cells were completely wiped from the top surface of the membrane. We measured migrating cells adhering to the undersurface of the filters by DAPI staining (Vector) quantified with Kodak 1D software. Results are indicative of four independent experiments.

Statistical analysis. Data are expressed as the mean ± s.e.m. Data were analyzed with an unpaired two-tailed Student's *t*-test or analysis of variance (ANOVA) coupled with posthoc Tukey's test for multiple pairwise comparisons. Probability values of $P < 0.05$ were considered to be statistically significant.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGEMENTS

We thank L.E. Huang and G.L. Semenza for HIF plasmids, and R. Schneider and E. Fisher for critically reviewing the manuscript. This study was supported by the National Institutes of Health/National Institute for Biomedical Imaging and Bioengineering (grant EB002265 to G.C.G.), the Sarnoff Endowment for Cardiovascular Research (G.C.G) and the Juvenile Diabetes Research Foundation (O.M.T).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 9 April; accepted 2 June 2004
Published online at http://www.nature.com/naturemedicine/

1. Peled, A. *et al.* Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* **283**, 845–848 (1999).
2. Peled, A. *et al.* The chemokine SDF-1 stimulates integrin-mediated arrest of CD34$^+$ cells on vascular endothelium under shear flow. *J. Clin. Invest.* **104**, 1199–1211 (1999).
3. McGrath, K.E., Koniski, A.D., Maltby, K.M., McGann, J.K. & Palis, J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev. Biol.* **213**, 442–456 (1999).
4. Kollet, O. *et al.* HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34$^+$ stem cell recruitment to the liver. *J. Clin. Invest.* **112**, 160–169 (2003).
5. Askari, A.T. *et al.* Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* **362**, 697–703 (2003).
6. Yamaguchi, J. *et al.* Stromal cell-derived factor-1 effects on *ex vivo* expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* **107**, 1322–1328 (2003).
7. Hitchon, C. *et al.* Hypoxia-induced production of stromal cell-derived factor 1 (CXCL12) and vascular endothelial growth factor by synovial fibroblasts. *Arthritis Rheum.* **46**, 2587–2597 (2002).
8. Tepper, O.M. *et al.* Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* **106**, 2781–2786 (2002).
9. Amara, A. *et al.* Stromal cell-derived factor-1α associates with heparan sulfates through the first β-strand of the chemokine. *J. Biol. Chem.* **274**, 23916–23925 (1999).
10. Semenza, G.L. Targeting HIF-1 for cancer therapy. *Nat. Rev. Cancer* **3**, 721–732 (2003).
11. Huang, L.E., Gu, J., Schau, M. & Bunn, H.F. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* **95**, 7987–7992 (1998).
12. Forsythe, J.A. *et al.* Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol. Cell. Biol.* **16**, 4604–4613 (1996).
13. Campbell, J. *et al.* Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* **279**, 381–384 (1998).
14. Reiss, K., Mentlein, R., Sievers, J. & Hartmann, D. Stromal cell-derived factor 1 is secreted by meningeal cells and acts as chemotactic factor on neuronal stem cells of the cerebellar external granular layer. *Neuroscience* **115**, 295–305 (2002).
15. Ratajczak, M.Z. *et al.* Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the presence of both muscle progenitors in bone marrow and hematopoietic stem/progenitor cells in muscles. *Stem Cells* **21**, 363–371 (2003).
16. Kawamoto, A. *et al.* Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. *Circulation* **103**, 634–637 (2001).
17. Asahara, T. *et al.* Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964–967 (1997).
18. Hill, J.M. *et al.* Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N. Engl. J. Med.* **348**, 593–600 (2003).
19. Signoret, N. *et al.* Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J. Cell Biol.* **139**, 651–664 (1997).
20. Sweeney, E., Lortat-Jacob, H., Priestley, G., Nakamoto, B. & Papayannopoulou, T. Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells. *Blood* **99**, 44–51 (2002).
21. Hattori, K. *et al.* Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. *Blood* **97**, 3354–3360 (2001).
22. Nagasawa, T. *et al.* Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* **382**, 635–638 (1996).
23. Zou, Y., Kottmann, A., Kuroda, M., Taniuchi, I. & Littman, D. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595–599 (1998).
24. Harrison, J.S., Rameshwar, P., Chang, V. & Bandari, P. Oxygen saturation in the bone marrow of healthy volunteers. *Blood* **99**, 394 (2002).
25. Staller, P. *et al.* Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. *Nature* **425**, 307–311 (2003).
26. Elson, D.A. *et al.* Induction of hypervascularity without leakage or inflammation in transgenic mice overexpressing hypoxia-inducible factor-1α. *Genes Dev.* **15**, 2520–2532 (2001).
27. Rivard, A. *et al.* Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. *J. Biol. Chem.* **275**, 29643–29647 (2000).
28. Muller, A. *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**, 50–56 (2001).
29. Takahashi, T. *et al.* Ischemia- and cytokine-induced mobilization of bone marrow–derived endothelial progenitor cells for neovascularization. *Nat. Med.* **5**, 434–438 (1999).
30. Dimmeler, S. *et al.* HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J. Clin. Invest.* **108**, 391–397 (2001).

Immunity, Vol. 19, 257–267, August, 2003, Copyright ©2003 by Cell Press

Long-Term Hematopoietic Stem Cells Require Stromal Cell-Derived Factor-1 for Colonizing Bone Marrow during Ontogeny

Toshiaki Ara,[1,2] Koji Tokoyoda,[1]
Tatsuki Sugiyama,[1,2] Takeshi Egawa,[2]
Kenji Kawabata,[2] and Takashi Nagasawa[1,3]
[1]Department of Medical Systems Control
Institute for Frontier Medical Sciences
Kyoto University
53 Kawahara-cho, Shogoin, Sakyo-ku
Kyoto 606-8507
Japan
[2]Department of Immunology
Research Institute
Osaka Medical Center for Maternal and Child Health
840 Murodo-cho, Izumi
Osaka 594-1101
Japan

Summary

The physiological role of SDF-1 on hematopoietic stem cells (HSCs) remains elusive. We show that colonization of bone marrow by HSCs in addition to myeloid cells is severely impaired in SDF-1$^{-/-}$ embryos by a long-term repopulation assay. Colonization of spleen by HSCs was also affected, but to a lesser extent. Enforced expression of SDF-1 under the control of vascular-specific Tie-2 regulatory sequences could completely rescue the reduction of HSCs but not myeloid cells in SDF-1$^{-/-}$ bone marrow. SDF-1 was detected in the vicinity of the vascular endothelial cells in fetal bone marrow. SDF-1 plays a critical role in colonization of bone marrow by HSCs and myeloid cells during ontogeny, and the mechanisms by which SDF-1 functions are distinct between HSCs and myeloid cells.

Introduction

Hematopoietic stem cells (HSCs) are defined as cells with the ability to supply all the various types of blood cells throughout life (Weissman, 2000). HSCs are mobile and sequence of hematopoietic colonization events occurs during ontogeny. It has been assumed previously that the first HSCs are generated in the AGM (aorta, gonad, and mesonephros) region and move to the fetal liver, the principal hematopoietic organ during embryogenesis (Cumano et al., 1996; Medvinsky and Dzierzak, 1996). Later, HSCs migrate to colonize the spleen and bone marrow, which is the primary hematopoietic site throughout adult life. In this process, HSCs may transmigrate across the endothelium to reach a putative hematopoietic stem cell niche in bone marrow or spleen. These colonizations of hematopoietic organs by HSCs are systematic and believed to be controlled by adhesion receptors and cytokines. The experiments using neutralizing antibodies suggested the importance of adhesion receptors. Treatment of antibodies against VLA-4 ($\alpha4\beta1$ integrin), $\beta1$ integrin, VCAM-1, or CD44 prevented homing of hematopoietic progenitors to adult bone marrow and spleen (Papayannopoulou et al., 1995; Vermeulen et al., 1998; Williams et al., 1991). However, genetic deletion of VCAM-1 or CD44 gene did not affect hematopoiesis in bone marrow and spleen (Friedrich et al., 1996; Schmits et al., 1997) and $\alpha4$ integrin-deficient multipotent progenitors developed normally in fetal liver, bone marrow, and spleen (Arroyo et al., 1996, 1999), suggesting that these molecules are dispensable for homing of HSCs. In contrast, it has been shown that $\beta1$ integrin-deficient hematopoietic cells were unable to seed fetal liver, bone marrow, spleen, and thymus, indicating that $\beta1$ integrins are critical for the colonization of all fetal and adult hematopoietic organs (Hirsch et al., 1996; Potocnik et al., 2000). Selectins have been shown to be essential for leukocyte homing to peripheral lymph nodes in health and disease. In mice deficient in both P- and E-selectins, bone marrow myelopoiesis was increased but homing of hematopoietic progenitors into bone marrow after irradiation was impaired (Frenette et al., 1996, 1998). Thus their physiological roles in hematopoietic colonization events remain unclear.

Among a variety of hematopoietic cytokines, only the chemokine, stromal cell-derived factor-1/pre-B-cell growth-stimulating factor (SDF-1/PBSF), has been shown to be involved in hematopoietic colonization during embryogenesis (Nagasawa et al., 1996a; Nagasawa, 2000). Chemokines are a large family of structurally related chemoattractive cytokines, which act via hepta helical receptors coupled to heterotrimeric GTP binding proteins (Baggiolini et al., 1997). SDF-1 was isolated from bone marrow stromal cell lines and first characterized as a pre-B cell growth-stimulating factor (Nagasawa et al., 1994, 1999). CXCR4 has been shown to be a primary physiologic receptor for SDF-1 (Bleul et al., 1996; Ma et al., 1998; Nagasawa et al., 1996a, 1996b; Oberlin et al., 1996; Tachibana et al., 1998; Zou et al., 1998) and also function as an entry coreceptor for strains of HIV-1 (Feng et al., 1996; Nagasawa et al., 1998). The multiple essential functions of SDF-1 and CXCR4 in development have been demonstrated using mutant mice with targeted gene disruption (Nagasawa et al., 1996a; Tachibana et al., 1998; Zou et al., 1998). SDF-1 and CXCR4 are essential for B cell development, blood vessel formation in gastrointestinal tract, cardiac ventricular septum formation, cerebellar development, and embryonic viability (Ma et al., 1998; Nagasawa et al., 1996a; Tachibana et al., 1998; Zou et al., 1998). In B cell development, it has been shown that dependency on SDF-1 appears at the earliest stages in both fetal liver and bone marrow (Egawa et al., 2001). Of particular note, SDF-1$^{-/-}$ or CXCR4$^{-/-}$ embryos have much more impaired myelopoiesis in bone marrow compared with myelopoiesis in fetal liver, suggesting that SDF-1 and CXCR4 are involved in colonization of bone marrow by hematopoietic progenitors during embryogenesis (Ma et al., 1998; Nagasawa et al., 1996a; Tachibana et al., 1998; Zou et al., 1998). However, considering that erythropoiesis was less affected in CXCR4$^{-/-}$ embryonic marrow (Zou et

[3]Correspondence: tnagasa@frontier.kyoto-u.ac.jp

al., 1998), there is the possibility that HSCs are not affected but instead homing or expansion of myeloid and B lymphocyte precursors is impaired in SDF-1$^{-/-}$ or CXCR4$^{-/-}$ bone marrow. It has been shown that human CD34^{+} hematopoietic progenitor cells from bone marrow or fetal blood migrate in response to SDF-1 in vitro and that plasma elevation of SDF-1 induces increase in murine hematopoietic progenitors in the peripheral blood, supporting a role for SDF-1 in mobilization of primitive hematopoietic progenitors (Aiuti et al., 1997; Hattori et al., 2001; Kim and Broxmeyer, 1998; Peled et al., 1999a; Sweeney et al., 2002). Experiments using radiation chimeras with lethally irradiated adult mice revealed a modest reduction in long-term myeloid reconstitution by CXCR4$^{-/-}$ fetal liver cells (Kawabata et al., 1999; Ma et al., 1999). In addition, it has been shown that treatment of immature human bone marrow CD34^{+} cells with anti-CXCR4 antibodies prevents short-term engraftment of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Peled et al., 1999). However, it is uncertain whether the reduction is due to the defects in HSCs or more mature progenitors in these transplantation experiments. On the other hand, it has been reported that short-term bone marrow engraftment is unaffected by treatment of bone marrow cells with pertussis toxin, an inhibitor of signaling by receptors coupled to heterotrimeric GTP binding proteins including chemokine receptors (Wiesmann et al., 1999). Thus physiological roles of SDF-1 and CXCR4 in colonization of hematopoietic organs by HSCs remain unclear, despite these studies. Here we measured the numbers of HSCs capable of long-term multilineage reconstitution of adults, in fetal hematopoietic organs from control and SDF-1$^{-/-}$ embryos, based on a competitive repopulation assay (Ema et al., 2000; Harrison et al., 1993; Micklem et al., 1972). In addition, to determine the functions of SDF-1 in the vicinity of endothelial cells, we examined the consequence of enforced expression of SDF-1 in the endothelial lineage cells in SDF-1$^{-/-}$ embryos. We have shown that the numbers of HSCs as well as myeloid cells are severely reduced in the bone marrow from SDF-1$^{-/-}$ embryos and that enforced expression of SDF-1 under the control of vascular-specific Tie-2 regulatory sequences in SDF-1$^{-/-}$ embryos completely rescues the reduction of HSCs but not myeloid cells in their bone marrow. Our results suggest that mechanisms by which SDF-1 controls seeding of bone marrow by HSCs and myeloid cells are distinct. These findings reveal a critical role for SDF-1 in colonization of bone marrow by HSCs during ontogeny and provide a molecular basis for exploring the key components of stem cell niches in bone marrow.

Results

Colonization of Bone Marrow by HSCs Is Severely Impaired in SDF-1$^{-/-}$ Embryos

At later developmental stages, HSCs home to and colonize the spleen and bone marrow, which is the primary hematopoietic site throughout adult life. It has been shown previously that SDF-1-deficient embryos, which die at or shortly before birth, reveal the defects in myelopoiesis and B lymphopoiesis in bone marrow (Nagasawa et al., 1996a). We analyzed hematopoiesis including erythropoiesis in fetal bone marrow from control or SDF-1$^{-/-}$ embryos by flow cytometry, using the B lymphoid markers B220 and CD19 and myeloid marker Gr-1 and erythroid marker Ter119. In E18.5 SDF-1$^{-/-}$ bone marrow, the numbers of Gr-1^{+} and B220^{+}CD19^{+} cells were severely reduced but the numbers of Ter119^{+} cells were normal or modestly reduced compared to control embryos (Figure 1A). These results raise the possibility that the mutation in SDF-1 does not affect HSCs that generate all hematopoietic lineage cells including erythroid cells but instead affects myeloid or B lymphoid precursors in fetal bone marrow. Thus we were prompted to examine directly the colonization of bone marrow by HSCs in SDF-1$^{-/-}$ embryos. HSCs are capable of long-term multilineage reconstitution of adults. The only completely unambiguous measure of HSCs is long-term transplantation. Thus, we estimated the numbers of HSCs using repopulating units (RU), based on a competitive repopulation assay (Ema et al., 2000; Harrison et al., 1993; Micklem et al., 1972), in hematopoietic organs from control or SDF-1$^{-/-}$ embryos. We could measure the numbers of RU in SDF-1$^{-/-}$ embryos since SDF-1$^{-/-}$ cells could receive the signal normally from SDF-1 produced by recipient mice when transferred to lethally irradiated wild-type mice (Kawabata et al., 1999). First, we examined the numbers of HSCs in fetal liver from control and SDF-1$^{-/-}$ embryos. HSCs are thought to move from fetal liver to bone marrow or spleen during embryogenesis. HSC activities were found in wild-type fetal liver from E12.5 onward (Figure 2A). Consistent with the previous results that erythropoiesis and myelopoiesis in fetal livers were less affected in SDF-1$^{-/-}$ embryos (Nagasawa et al., 1996a; Egawa et al., 2001), the numbers of HSCs appeared normal in SDF-1$^{-/-}$ fetal livers from E12.5 to E14.5 (Figure 2A). Subsequently, the numbers of HSCs in E16.5 SDF-1$^{-/-}$ fetal livers were reduced approximately 2-fold compared with control livers, and the HSC numbers were similar in control and mutant embryos at E18.5 (Figure 2A). Next, we evaluated the numbers of HSCs in bone marrow from control and SDF-1$^{-/-}$ embryos. In E18.5 bone marrow, HSCs were readily detected in SDF-1$^{+/+}$ or SDF-1$^{+/-}$ embryos by a long-term competitive repopulation assay (Figure 2B). In contrast, HSCs were severely decreased in bone marrow from E18.5 SDF-1$^{-/-}$ embryos (Figure 2B). Spleen is also a hematopoietic site throughout life in the mouse. Flow cytometric analysis revealed the reduction in the numbers of myeloid and B lymphocyte lineage cells in E18.5 SDF-1$^{-/-}$ spleen compared with control spleen while the reductions were less severe than the reductions in bone marrow (Figure 1B). Consistent with this, the numbers of HSCs were also affected in the E18.5 SDF-1$^{-/-}$ spleen, but to a lesser extent than those in bone marrow (Figure 2C).

It has been shown previously that the myeloid cells and B cell precursors in peripheral blood were increased in CXCR4$^{-/-}$ embryos compared with wild-type embryos (Ma et al., 1999). We measured the numbers of HSCs in fetal peripheral blood from control and SDF-1$^{-/-}$ embryos during embryogenesis. As shown in Figure 2D, the numbers of HSCs were greatly increased in the peripheral blood from E14.5, E16.5, and E18.5 SDF-1$^{-/-}$ embryos compared with control embryos (Figure 2D).

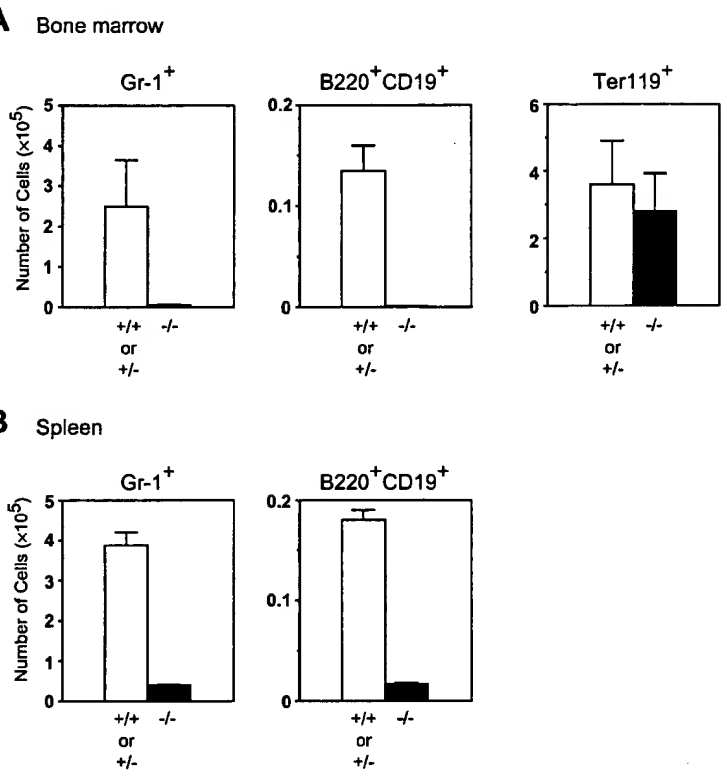


Figure 1. Hematopoiesis in E18.5 Bone Marrow or Spleen from Control or SDF-1$^{-/-}$ Embryos

Flow-cytometric analysis of E18.5 bone marrow cells or spleen cells stained with anti-Ter119, anti-Gr-1, anti-B220, and anti-CD19. The numbers of Ter119^{+} erythroid cells, Gr-1^{+} myeloid cells, and B220^{+}CD19^{+} B lymphoid cells were quantified per embryo in bone marrow (A) or spleen (B).

Since SDF-1 has been shown to be essential for vascularization of gastrointestinal tracts (Tachibana et al., 1998), there is the possibility that SDF-1$^{-/-}$ embryos have deficits in bone marrow blood vessel formation that affect hematopoiesis. Then we examined the vasculature in fetal bone marrow from wild-type and SDF-1$^{-/-}$ embryos by whole-mount immunohistostains using an antibody to CD31 (PECAM-1), a marker expressed on all endothelial cells. There was no significant difference in the vasculature of E18.5 bone marrow between the wild-type and SDF-1$^{-/-}$ embryos (Figure 3).

Enforced Expression of SDF-1 in Vascular Endothelial Cells Rescues the Colonization of Bone Marrow by HSCs but Not by Myeloid Cells in SDF-1$^{-/-}$ Embryos

Colonization of hematopoietic organs by hematopoietic cells is thought to occur by multistep processes. Initially, emigration of hematopoietic cells from the vasculature into hematopoietic tissues requires them to roll along the endothelial cells, adhere firmly to endothelial cells, and transmigrate through the endothelium. Thereafter, hematopoietic cells migrate and adhere to the stem cell niches that support their survival, proliferation, and differentiation. Thus vascular endothelial cells are thought to be the key elements responsible for the hematopoietic colonization events and we speculated that SDF-1 in the vicinity of the endothelial cells might play a role in the colonization of bone marrow by HSCs. To address this possibility, we introduced SDF-1 transgene that was expressed in endothelial cells into SDF-1$^{-/-}$ embryos. Initially, we generated transgenic mice expressing the SDF-1 gene under the control of the *cis*-acting sequences of the Tie-2 gene (Tie-2 SDF-1 transgenic mice) (Schlaeger et al., 1997). Tie-2 is a vascular endothelial-specific receptor tyrosine kinase and this transcriptional enhancer sequences allow it to target reporter gene expression specifically and uniformly to virtually all vascular endothelial cells throughout embryogenesis and adulthood (Schlaeger et al., 1997). Tie-2 SDF-1 transgenic mice were healthy and fertile, and crossed with SDF-1$^{+/-}$ mice and resultant progeny (Tie-2 SDF-1/SDF-1$^{+/-}$) were backcrossed with SDF-1$^{+/-}$ mice. The Tie-2 SDF-1/SDF-1$^{-/-}$ mice were embryonic lethal as were SDF-1$^{-/-}$ mice. Whole-mount immunohistochemical analysis of Tie-2 SDF-1/SDF-1$^{-/-}$ embryos was performed and revealed the same defects in formation of the large vessels supplying the gastrointestinal tract as those observed in SDF-1$^{-/-}$ embryos (data not shown), suggesting that enforced expression of SDF-1 in endothelial cells did not affect blood vessel formation in the mutants. Next, we evaluated the numbers of HSCs using RU, based on a competitive repopulation assay in E18.5 fetal liver, bone marrow, spleen, and peripheral blood from litters including SDF-1$^{+/-}$, Tie-2 SDF-1/SDF-1$^{+/-}$, SDF-1$^{-/-}$, and Tie-2 SDF-1/SDF-1$^{-/-}$ embryos. In fetal liver, the numbers of HSCs were similar between SDF-1$^{+/-}$, Tie-2 SDF-1/SDF-1$^{+/-}$, SDF-1$^{-/-}$, and Tie-2 SDF-1/SDF-1$^{-/-}$ embryos (Figure 4A). In contrast, there was a significant increase in the numbers of HSCs in bone marrow from Tie-2 SDF-1/SDF-1$^{-/-}$ embryos compared with SDF-1$^{-/-}$ embryos as shown in Figure 4A. These results demonstrated that expression of SDF-1 under the control of vascular-specific Tie-2 regulatory sequences completely rescued the colonization of bone marrow by HSCs in SDF-1$^{-/-}$ embryos. In addition, the

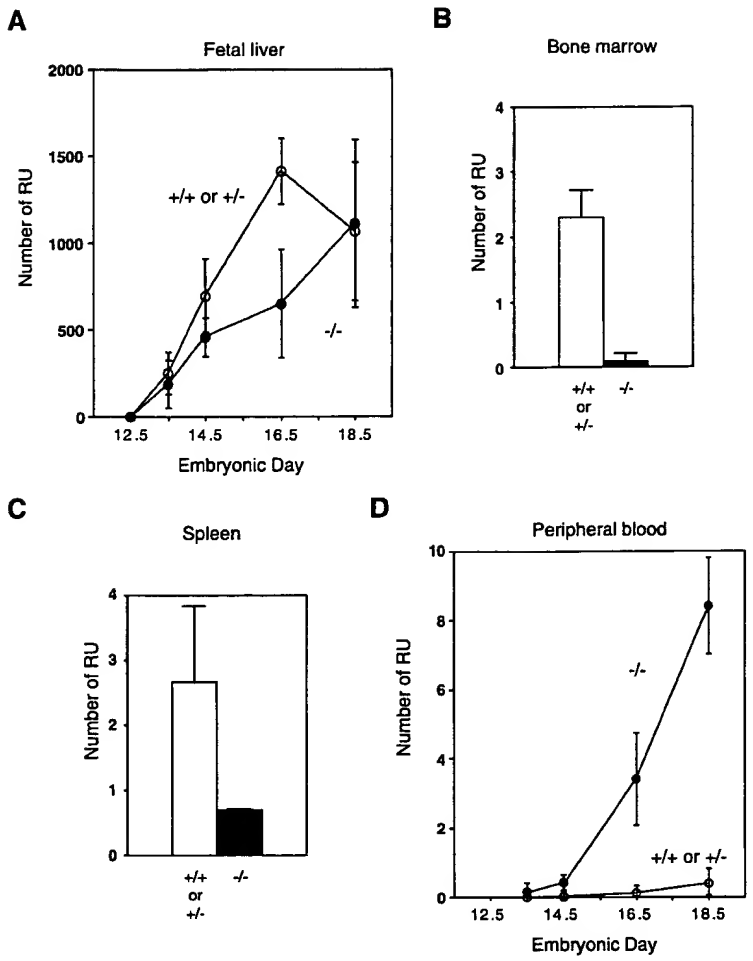


Figure 2. Long-Term Hematopoietic Stem Cells in Hematopoietic Organs from Control and SDF-1$^{-/-}$ Embryos

The numbers of HSCs were estimated using repopulating unit (RU), based on a competitive repopulation assay in fetal liver, bone marrow, spleen, or peripheral blood. Lethally irradiated Ly5.1 mice were coinjected with test Ly5.2 cells from hematopoietic organs and a constant dose of competitor Ly5.1 bone marrow. At 16 weeks after transplantation, recipient mice were analyzed for the contribution to bone marrow cells from test and competitor populations by flow cytometric analysis. The numbers of RU were expressed per embryo in fetal liver (A), bone marrow (B), spleen (C), or peripheral blood (D) at different developmental time points (A and D) or at E18.5 (B and C).

numbers of HSCs were significantly increased in the spleen from Tie-2 SDF-1/SDF-1$^{-/-}$ embryos compared with SDF-1$^{-/-}$ embryos (Figure 4A). As shown above, the numbers of HSCs in peripheral blood were increased in SDF-1$^{-/-}$ embryos compared with control embryos at E18.5 (Figure 2D). However, enforced expression of SDF-1 in endothelial cells did not significantly decrease the HSC numbers in SDF-1$^{-/-}$ peripheral blood (Figure

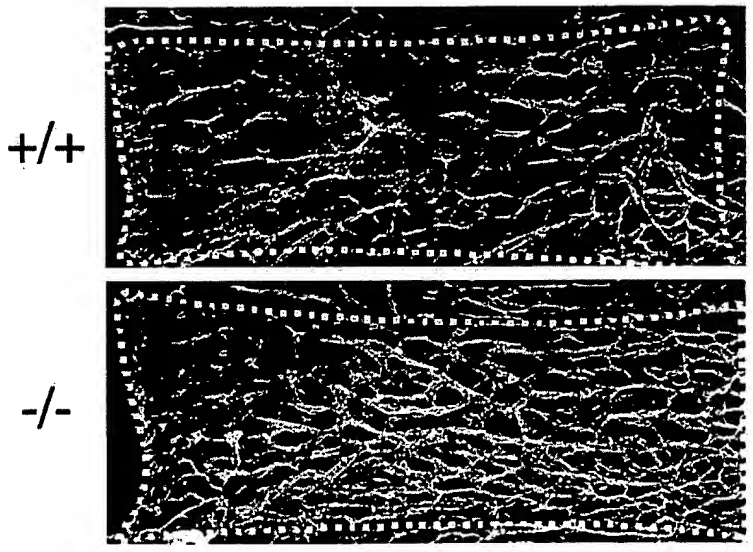


Figure 3. Normal Patterning of Vessels in Bone Marrow from SDF-1$^{-/-}$ Embryos

Whole-mount view of E18.5 wild-type (upper) and SDF-1$^{-/-}$ (lower) bone marrow stained with an antibody to PECAM-1, a marker expressed on all endothelial cells. Dashed line, approximate border of bone marrow cavity.

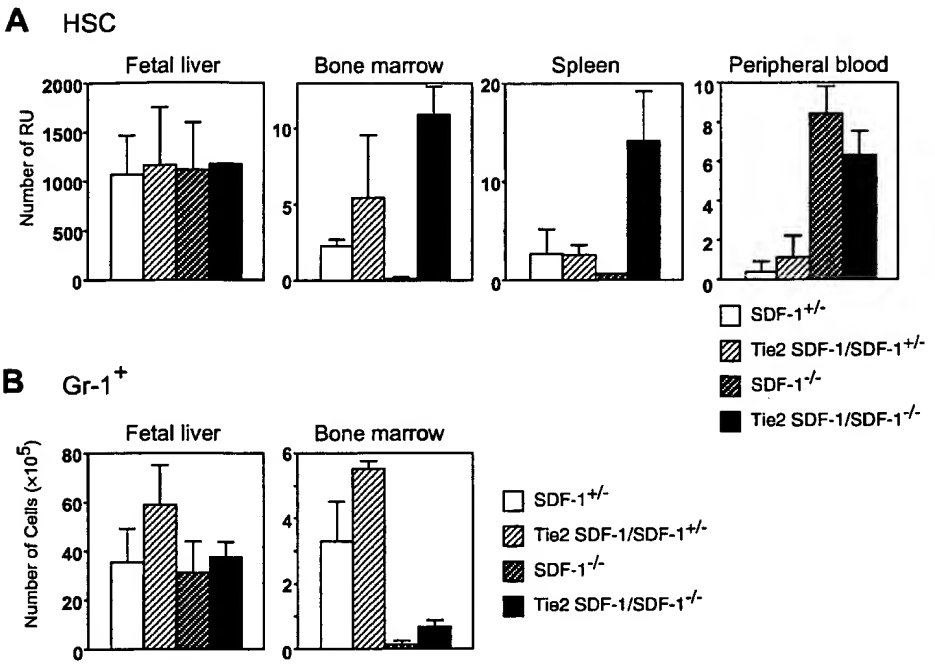


Figure 4. Enforced Expression of SDF-1 in Endothelial Cells Rescues the Colonization of Bone Marrow by HSCs but Not by Myeloid Cells

(A) Long-term hematopoietic stem cells (HSCs) in E18.5 hematopoietic organs from SDF-$1^{+/-}$, Tie-2 SDF-1/SDF-$1^{+/-}$, SDF-$1^{-/-}$, and Tie-2 SDF-1/SDF-$1^{-/-}$ embryos. The numbers of HSCs were estimated using repopulating unit (RU), based on a competitive repopulation assay in fetal liver, bone marrow, spleen, or peripheral blood. Lethally irradiated Ly 5.1 mice were coinjected with test Ly5.2 cells from hematopoietic organs and a constant dose of competitor Ly5.1 bone marrow. At 16 weeks after transplantation, recipient mice were analyzed for the contribution to bone marrow cells from test and competitor populations by flow cytometric analysis. The numbers of RU were expressed per embryo in fetal liver, bone marrow, spleen, or peripheral blood.

(B) Myelopoiesis in E18.5 bone marrow from SDF-$1^{+/-}$, Tie-2 SDF-1/SDF-$1^{+/-}$, SDF-$1^{-/-}$, and Tie-2 SDF-1/SDF-$1^{-/-}$ embryos. Flow-cytometric analysis of E18.5 fetal liver or bone marrow cells stained with anti-Gr-1. The numbers of Gr-1^+ myeloid cells were quantified per embryo in fetal liver and bone marrow.

4A), indicating that accumulation of HSCs in peripheral blood in SDF-$1^{-/-}$ embryos was not due to defective colonization of bone marrow and spleen by HSCs.

Since SDF-$1^{-/-}$ embryos show active myelopoiesis in fetal liver, the defects observed in myelopoiesis in bone marrow from SDF-$1^{-/-}$ mice could not be due to the defective myelopoiesis but instead due to the defective colonization of bone marrow by HSCs. However, surprisingly, flow cytometric analysis revealed that the numbers of Gr-1^+ myeloid cells were significantly reduced in the bone marrow from E18.5 Tie-2 SDF-1/SDF-$1^{-/-}$ embryos compared with Tie-2 SDF-1/SDF-$1^{+/-}$ or SDF-$1^{+/-}$ embryos, indicating that defective myelopoiesis in SDF-$1^{-/-}$ fetal bone marrow were not rescued by the enforced expression of SDF-1 in endothelial cells (Figure 4B). Furthermore, enforced expression of SDF-1 under the control of vascular-specific Tie-2 regulatory sequences did not affect the myelopoiesis in fetal livers from SDF-$1^{-/-}$ embryos (Figure 4B).

Myeloid Cells in Fetal Bone Marrow and Peripheral Blood Are Predominantly Nonproliferating Relative to Fetal Liver, and Require CXCR4 for Colonizing Bone Marrow

The result that expression of SDF-1 under the control of vascular-specific Tie-2 regulatory sequences in SDF-$1^{-/-}$ embryos rescued the colonization of bone marrow by HSCs but not by myeloid cells raises the possibility that myeloid cells in fetal bone marrow are not derived from HSCs that reside within bone marrow. To address this issue, we first examined the degree of proliferating cells in Gr-1^+ myeloid cells from wild-type fetal liver, bone marrow, and peripheral blood. In bone marrow, Gr-1^+ cells were first detectable at significant levels at E16.5 and thereafter increased from E16.5 to E18.5 (data not shown). Sorted Gr-1^+cells were analyzed by flow cytometry for DNA content by propidium iodide staining. The cells in cycle in Gr-1^+ cells from E18.5 bone marrow or peripheral blood were almost absent although E18.5 Gr-1^+ fetal liver cells contained significant numbers of cells in cycle (Figure 5A). These results indicate that Gr-1^+ cells in bone marrow or peripheral blood are predominantly nonproliferating although fetal liver contains a significant proportion of proliferating Gr-1^+ cells. Next, we compared the expression of CXCR4 in Gr-1^+ myeloid cells from E18.5 fetal liver, bone marrow, and peripheral blood by flow cytometry using an anti-mouse CXCR4 mAb. CXCR4 expression in Gr-1^+ cells from bone marrow was similar to peripheral blood Gr-1^+ cells but significantly lower compared to fetal liver Gr-1^+ cells (Figure 5B). These data suggest that most of the Gr-1^+ cells in fetal bone marrow are not developing within bone marrow but are instead derived from the peripheral circulation. This prompted us to test directly the roles of SDF-1

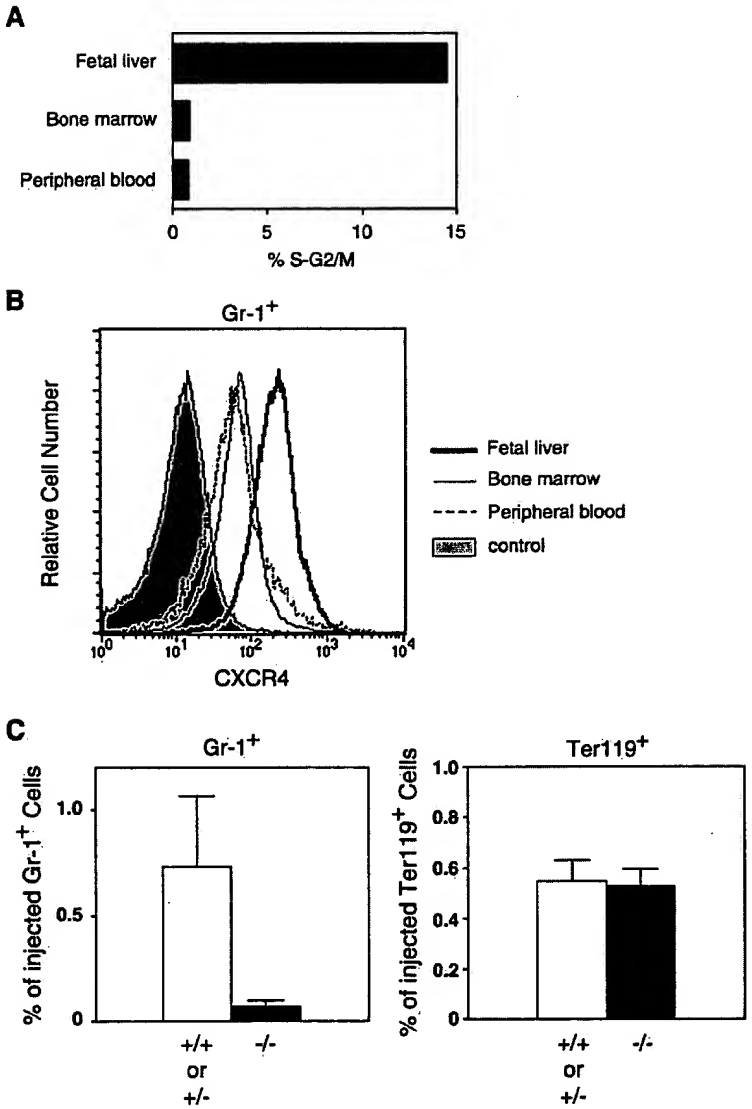


Figure 5. Gr-1$^+$ Myeloid Cells in Fetal Bone Marrow and Peripheral Blood Were Predominantly Nonproliferating Relative to Fetal Liver and Require CXCR4 for Colonizing Bone Marrow

(A) Cell cycle analysis of Gr-1$^+$ cells in E18.5 fetal liver, bone marrow, and peripheral blood. Cells were stained with anti-Gr-1 and then 10^5 cells were sorted, fixed, stained with propidium iodide, and analyzed by quantitative flow cytometry. The percentage of S+G2/M phase cells is displayed.

(B) Cell surface expression of CXCR4 on Gr-1$^+$ cells in E18.5 fetal liver, bone marrow, and peripheral blood. Cells were stained with antibodies against CXCR4 and Gr-1 and then analyzed on a flow cytometer. Gr-1$^+$ cells in bone marrow or peripheral blood have lower levels of CXCR4 in contrast with the Gr-1$^+$ cells in fetal liver.

(C) Impaired homing of CXCR4-deficient Gr-1$^+$ cells into bone marrow. Approximately 7×10^6 (left) or 2×10^6 (right) calcein-AM-labeled cells derived from wild-type or CXCR4$^{-/-}$ fetal livers were intravenously injected into wild-type recipients. After 16 hr (left) or 4 hr (right), cells were isolated from bone marrow of recipients and analyzed by flow cytometry using anti-Gr-1 or anti-Ter119, respectively. The graph shows the donor Gr-1$^+$ or Ter119$^+$ cells populating bone marrow as a percentage of injected Gr-1$^+$ or Ter119$^+$ cells to compensate for the initial difference in the numbers of fetal liver Gr-1$^+$ or Ter119$^+$ cells, respectively.

in emigration of the Gr-1$^+$ cells into bone marrow. We transferred calcein-AM-labeled Gr-1$^+$ or Ter119$^+$ cells derived from wild-type or CXCR4$^{-/-}$ fetal livers into wild-type recipients. Upon sixteen hours of transfer, Gr-1$^+$ cells derived from E18.5 wild-type embryos had migrated to the bone marrow of recipient animals. In contrast, Gr-1$^+$ cells derived from CXCR4$^{-/-}$ embryos failed to colonize recipient bone marrow (Figure 5C). On the other hand, E14.5 CXCR4$^{-/-}$ Ter119$^+$ cells had entered the recipients' bone marrow with a similar frequency to that observed after transfer of wild-type cells (Figure 5C). Together, SDF-1 is likely to play an essential role in homing of Gr-1$^+$ cells into bone marrow from the peripheral circulation and enforced expression of SDF-1 in vascular endothelial cells does not rescue the defective colonization of bone marrow by Gr-1$^+$ cells in SDF-1$^{-/-}$ embryos.

SDF-1 Is Located in the Vicinity of the Vascular Endothelial Cells in Fetal Bone Marrow

It has been reported that SDF-1 is expressed in spindle-shaped stromal cells (Nagasawa et al., 1996), microvessels (Peled et al., 1999; Ponomaryov et al., 2000), or osteoblastic cells (Ponomaryov et al., 2000) in adult or fetal bone marrow. To study further the localization of SDF-1 within fetal bone marrow, two approaches were utilized. First, we generated the mice in which the GFP reporter gene was knocked into the SDF-1 locus (SDF-1/GFP knockin mice). The bone marrow from E18.5 SDF-1/GFP embryos were stained with a mAb to the pan-endothelial marker PECAM-1 to compare the distribution of SDF-1/GFP expressing cells with the distribution of endothelial cells. The blood vessels including medullary arteries, the network of medullary sinusoids, and venous sinus were observed and SDF-1/GFP expression was seen along the blood vessels in the E18.5 fetal bone marrow (Figure 6A). High magnification demonstrated that SDF-1/GFP expression was observed in mesenchymal cells in the vicinity of vascular endothelial cells but not in the endothelial cells per se (Figure 6B). Next we performed immunohistostaining using antibodies to SDF-1 and PECAM-1. In agreement with the analysis using SDF-1/GFP knockin mice, SDF-1 was observed in the vicinity of endothelial cells in extravascular spaces

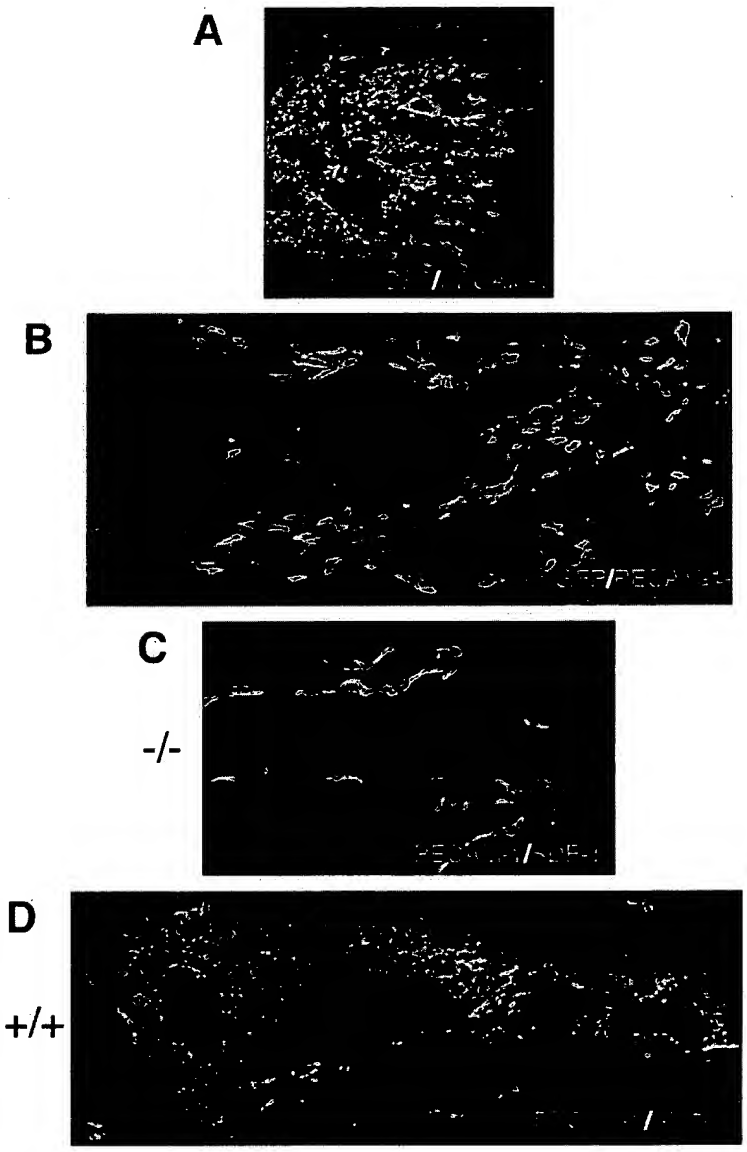


Figure 6. Localization of SDF-1 in Fetal Bone Marrow

(A and B) Whole-mount view of E18.5 bone marrow from knockin mice with one GFP marked SDF-1 allele, showing immunofluorescent staining with an antibody to pan-endothelial signal PECAM-1 (red) and SDF-1/GFP signal (green) at 10× magnification (A) and at 40× magnification (B). SDF-1-expressing cells were seen in the vicinity of endothelial cells in fetal bone marrow.

(C and D) Fluorescence and immunostaining microscopic analysis of E18.5 bone marrow sections from wild-type (D) or SDF-$1^{-/-}$ (C) embryos. Sagittal sections of fetal bone marrow stained with antibodies to SDF-1 (red) and PECAM-1 (green). E18.5 SDF-$1^{-/-}$ bone marrow was used as the negative control (C). Magnification, 40×. SDF-1 were detected in the vicinity of endothelial cells in fetal bone marrow.

in E18.5 wild-type fetal bone marrow. These findings are consistent with the notion that SDF-1 adjacent to endothelial cells plays a critical role in colonization of bone marrow by HSCs (Figures 6C and 6D).

Discussion

In this report, we analyzed the physiological role of SDF-1 in the colonization of hematopoietic organs by HSCs using a long-term competitive repopulation assay. HSCs are generated in AGM and expand in fetal liver. Then HSCs are released from hematopoietic organs including fetal liver and colonize bone marrow or spleen via the peripheral blood. A long-term competitive repopulation assay revealed that, in SDF-$1^{-/-}$ embryos, the numbers of HSCs were comparable in fetal liver and increased in peripheral blood but were severely reduced in bone marrow compared to control embryos (Figure 2). These results demonstrate that SDF-1 is essential for the colonization of bone marrow by HSCs from the peripheral circulation during embryogenesis. Furthermore, we revealed that enforced expression of SDF-1 in vascular endothelial cells could completely rescue the reduction of HSCs in SDF-$1^{-/-}$ bone marrow and spleen. The location and characterization of hematopoietic stem cell niches remain elusive. Our results suggest that endothelial cells could function as stromal environment essential for colonization of fetal bone marrow by HSCs in the presence of SDF-1. Together with the results that demonstrate the location of SDF-1 in the vicinity of endothelial cells (Figure 6), SDF-1 distributed in the mesenchyme surrounding endothelial cells and adjacent SDF-1 expressing stromal cells is likely to act as a key component of hematopoietic stem cell niches in fetal bone marrow.

Homing of HSCs into bone marrow is thought to occur by different processes including emigration from the vasculature into the stem cell niches through the endothelium, residing, survival, and self-renewing in the

niches. Considering that SDF-1 is chemotactic for hematopoietic stem or progenitor cells in vitro (Aiuti et al., 1997; Hattori et al., 2001; Kim and Broxmeyer, 1998; Peled et al., 1999a; Sweeney et al., 2002), it is possible that SDF-1 plays a role in extravasation and homing of HSCs into the stem cell niches. SDF-1 secreted from stromal cells adjacent to the endothelial cells may arrive in the vessel and regulate adhesion of HSCs to endothelial in bone marrow. In addition, there is also the possibility that SDF-1 plays a role in promoting survival or self-renewing of HSCs around endothelial cells in bone marrow. Previous studies revealed that SDF-1 supported the survival or proliferation of hematopoietic progenitors in vitro (Egawa et al., 2001; Hodohara et al., 2000; Lataillade et al., 2000, 2002; Nagasawa et al., 1994). In this regard, expansion of HSCs in fetal liver was modestly impaired in SDF-1$^{-/-}$ fetal liver from E14.5 to E16.5 (Figure 2A). On the other hand, in vitro studies using neutralizing antibodies against SDF-1 revealed a role of SDF-1 in autocrine survival of human peripheral blood CD34^{+}CD38^{+} more committed cells (Lataillade et al., 2002). Since it has been reported that Tie-2 is expressed on the HSCs (Hsu et al., 2000), there is also the possibility that enforced expression of SDF-1 in HSCs supports the survival of HSCs through an autocrine/paracrine manner and restores the colonization of bone marrow by HSCs in SDF-1$^{-/-}$ embryos. However, this possibility is less likely because enforced expression of SDF-1 did not increase the long-term repopulating ability for fetal liver cells (Figure 4A).

In contrast to HSCs, enforced expression of SDF-1 in endothelial cells could not rescue the reduction of myeloid cells in SDF-1$^{-/-}$ bone marrow during embryogenesis. These results indicate that endothelial cells cannot function as stromal environment that provided appropriate conditions for myeloid development in fetal bone marrow and suggest that defects in myelopoiesis in SDF-1$^{-/-}$ bone marrow are not due simply to the deficits in colonization by HSCs. Active lymphomyelopoiesis appears from E17.5 in bone marrow. First, HSCs homing to embryonic marrow are thought to give rise to hematopoietic precursors and mature blood cells during ontogeny. In this case, our results suggest that myelopoiesis requires the expression of SDF-1 in mesenchymal stromal cells in fetal bone marrow. SDF-1 may attract HSCs or committed precursors to the stromal cells and/or support their differentiation synergistically with stromal cell-derived molecules. Alternatively, it is possible that the circulation of lineage committed hematopoietic progenitors also plays a role in the seeding of the hematopoietic organs as previously suggested (Delassus and Cumano, 1996). Since myeloid cells in bone marrow and peripheral blood were predominantly nonproliferating relative to fetal liver and since levels of cell surface expression of CXCR4 in myeloid cells in bone marrow were similar to peripheral blood but low compared to fetal liver (Figures 5A and 5B), we hypothesize that the majority of hematopoietic cells in fetal bone marrow are derived from the peripheral circulation. Transfer experiments revealed that myeloid cells derived from CXCR4$^{-/-}$ fetal liver failed to colonize adult bone marrow (Figure 5C). Thus, SDF-1 would play a critical role in recruiting myeloid cells as well as HSCs from the peripheral circulation into bone marrow and enforced expression of SDF-1 in endothelial cells could restore the homing of HSCs but not myeloid cells. In this case, the cellular and molecular mechanisms of homing of HSCs and myeloid cells would be different. This hypothesis was called into question by the results that homing of HSC is severely impaired but erythropoiesis appears normal in SDF-1$^{-/-}$ bone marrow (Figures 1A and 2B). An explanation for this discrepancy is that absence of SDF-1 does not impair the emigration of erythroid cells from peripheral blood. Transfer experiments suggested that CXCR4 was not essential for the homing of fetal Ter119^{+} erythroid cells from circulation into adult bone marrow (Figure 5C). Whichever is the case, our findings indicate that mechanisms by which SDF-1 controls colonization of bone marrow by HSCs and myeloid cells are different.

HSCs also colonize the spleen as well as bone marrow at later developmental stages. The reduction in the numbers of HSCs in SDF-1$^{-/-}$ spleen was more modest than the reduction in SDF-1$^{-/-}$ bone marrow although enforced expression of SDF-1 in endothelial cells rescued the defects in colonization of spleen by HSCs in SDF-1$^{-/-}$ embryos. Therefore it is likely that SDF-1 plays a role in homing of HSCs to spleen but other cytokines can substitute in its absence. Thus our results suggest that molecular mechanisms that control stem cell colonization are different between bone marrow and spleen.

The numbers of HSCs in peripheral blood were increased in SDF-1$^{-/-}$ embryos (Figure 2D). Why are HSCs accumulated in the peripheral circulation in SDF-1$^{-/-}$ embryos? There are several possibilities. First, the numbers of HSCs that released from fetal liver, a major reservoir for HSCs during embryogenesis, were increased in SDF-1$^{-/-}$ embryos. Second, the numbers of HSCs that exited to the peripheral circulation were decreased in SDF-1$^{-/-}$ embryos. This latter possibility is unlikely because enforced expression of SDF-1 in endothelial cells restored the migration of HSCs from circulation into bone marrow and spleen but did not significantly alter the accumulation of HSCs in peripheral blood. Further studies will be needed to address this issue.

In contrast with bone marrow or spleen, the numbers of HSCs in fetal liver were similar in E18.5 SDF-1$^{+/-}$, Tie-2 SDF-1/SDF-1$^{+/-}$, SDF-1$^{-/-}$, and Tie-2 SDF-1/SDF-1$^{-/-}$ embryos (Figure 4A). Thus SDF-1 is dispensable for development of HSCs in fetal liver. However, since the numbers of HSCs in wild-type fetal liver have reached a maximum at E16.5 but those observed in SDF-1$^{-/-}$ fetal liver increased from E16.5 to E18.5 (Figure 2A), there is also the possibility that the expansion of HSCs in fetal liver is impaired or delayed in SDF-1$^{-/-}$ embryos. Whichever is the case, it is likely that enforced expression of SDF-1 in endothelial cells could not inhibit the release of HSCs from fetal liver or recruit HSCs from the peripheral circulation into fetal liver.

Chemokines have been shown to activate integrin functions in hematopoietic cells (Tanaka et al., 1993). Colonization of hematopoietic organs has been shown to require β1 integrins (Hirsch et al., 1996; Potocnik et al., 2000). However, there were phenotypic differences between mutants lacking SDF-1 and β1 integrins. While β1 integrin-deficient hematopoietic cells were unable to seed fetal liver, development of HSCs, erythroid, and myeloid cells were less affected in SDF-1$^{-/-}$ fetal livers.

How SDF-1 regulates the functions of integrins in colonization of bone marrow by HSCs is an important question for the future.

It had been thought that colonization of bone marrow by HSCs occurred only during embryogenesis or in pathological cases where there was more demand for hematopoiesis. However, it has been shown that HSCs constitutively migrate through the blood and play a physiological role in the functional reengraftment of unconditioned adult bone marrow (Wright et al., 2001). Thus it will be interesting to clarify the role of SDF-1 in the physiological migration of HSCs throughout adult life.

Together, the data presented here reveal that SDF-1 is essential for colonization of bone marrow by HSCs during ontogeny and define SDF-1 as a key component of hematopoietic stem cell niches. This study has provided an invaluable window into the molecular basis of hematopoietic colonization events by stem cells.

Experimental Procedures

Mice

The generation of SDF-1$^{-/-}$ and CXCR4$^{-/-}$ mice has been described previously (Nagasawa et al., 1996a; Tachibana et al., 1998). SDF-1$^{+/-}$ and CXCR4$^{+/-}$ mice were backcrossed more than ten times with C57BL/6-Ly5.2 mice. C57BL/6-Ly5.1 mice were a gift from Drs. M. Osawa and H. Nakauchi (University of Tokyo, Tokyo, Japan).

Generation of Transgenic Mice

Tie2 kinase promoter/enhancer (Schlaeger et al., 1997), released from pSPTg.T2FXK (a gift from T.N. Sato, The University of Texas, Dallas, Texas), rabbit β-globin intron, mouse polyadenylation signal sequences were introduced into pBlu-SDF-1/PBSF that contains murine SDF-1 cDNA. DNA was linealized and used for pronuclear microinjection to obtain Tie2 SDF-1 transgenic mice.

Generation of GFP Knockin Mice

To obtain SDF-1 directed GFP expression, exon 2 of the SDF-1 gene was replaced by GFP expression cassette by homologous recombination in ES cells (Nagasawa et al., 1996). Mutated ES colonies were used to produce mice hemizygous for the GFP insertion by blastocyst injection as described (Nagasawa et al., 1996a). Mice hemizygous for the GFP insertion that have one functional SDF-1 allele are phenotypically normal and can be used for the analysis of SDF-1 expression.

Cell Preparation

Fetal liver, bones, and spleen were isolated under a dissecting microscope (Leica). Fetal liver cells were suspended in D-MEM containing 2% fetal calf serum (FCS) through needles of 18- to 21-gauge. Fetal bone marrow cells were harvested from both sides of scapula, humerus, ulna, radius, femur, fibula, and tibia. Fetal bones and spleen were crushed between glass slides and resuspended in D-MEM containing 2% FCS. Fetal peripheral blood was collected by decapitating the embryo in D-MEM containing 2% FCS. Adult bone marrow cells from femurs were suspended in D-MEM containing 2% FCS by repeated flushing through a 21-gauge needle. Cell suspensions were filtered through nylon mesh to remove debris.

Staining, Flow Cytometry Analysis, and Cell Sorting

Antibodies used in this study were as follows: FITC-conjugated anti-Ly5.2 (clone: 104), anti-Ter119, anti-Gr-1 (RB6-8C5), and anti-B220 (RA3-6B2); PE-conjugated anti-Gr-1 (RB6-8C5) and anti-CD19 (1D3); biotinylated anti-CXCR4 (2B11), anti-Gr-1 (RB6-8C5) and isotype control IgG2bκ (A95-1). All antibodies were purchased from BD PharMingen (San Diego, CA).

Cells were stained with monoclonal antibodies for 20 min on ice. Subsequently, cells were washed, pelleted, and resuspended with APC-conjugated streptavidin (BD PharMingen) for 20 min. Stained cells were pelleted, resuspended in phosphate-buffered saline (PBS) containing 2% FCS, and analyzed with FACSCalibur (Becton Dickinson) or sorted with FACSVantageSE (Becton Dickinson). Dead cells were excluded by propidium iodide (PI) staining.

Competitive Repopulation Assay

The numbers of HSCs were estimated using repopulating unit (RU), based on a competitive repopulation assay (Ema et al., 2000; Harrison et al., 1993; Micklem et al., 1972). As Ly5.2 test cells, we used fetal liver cells (from E12.5 to E18.5), bone marrow cells, spleen cells, and peripheral blood cells. 1×10^6 fetal liver cells (from E13.5 to E18.5) were mixed with 2×10^6 cells of competitor Ly5.1 bone marrow. 1×10^6 (E12.5) fetal liver cells, a third of bone marrow cells and peripheral blood cells, and half of spleen cells were mixed with 1×10^6 cells of competitor Ly5.1 bone marrow. A cell mixture in 0.3 ml of D-MEM was injected intravenously into a group of recipient C57BL/6-Ly5.1 mice lethally irradiated at a single dose of 1,100 rads. At 16 weeks after transplantation, recipient mice were analyzed for the contribution to bone marrow cells from test and competitor populations by flow cytometric analysis. Percentage of donor-type in bone marrow myeloid cells, which are short-lived, were correlated to those in bone marrow B cell precursors and thus are a good indication for relative hematopoietic stem cell activity. RU was calculated using Harrison's method (Ema et al., 2000; Harrison et al., 1993; Micklem et al., 1972). If a test population contains 1 RU, it populates as well as 10^5 fresh competitor bone marrow cells.

Immunohistochemical Staining and Confocal Microscopy

For section staining, samples were fixed in 4% paraformaldehyde, equilibrated in 30% sucrose/PBS, and frozen. Then, sections were incubated with rat anti-PECAM-1 antibody (MEC13.3, BD PharMingen), rabbit anti-SDF-1 antibody (Torroy Pines Biolabs), Alexa488-conjugated goat anti-rat IgG (Molecular Probe, Eugene, OR), biotinylated-anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and Alexa546-conjugated streptavidin (Molecular Probe).

For whole-mount immunohistochemistry, fetal bones from SDF-1/GFP knockin mice were fixed in 4% paraformaldehyde, permeabilized with PBS containing 0.3% Triton X-100 (PBST), and blocked with PBST containing 1% skim milk (DIFCO). Then, tissues were stained with rat anti-PECAM-1 antibody and Alexa546-conjugated goat anti-rat IgG (Molecular Probe).

Tissues were mounted with Slowfade Antifade kit (Molecular Probe). All confocal microscopy was carried out on a LSM 5 PASCAL (Carl Zeiss).

Cell Cycle Analysis

Cell suspension incubated on ice for 30 min with FITC-conjugated anti-Gr-1 were washed with D-MEM containing 2% FCS and 0.01% NaN_3 before sorting in a FACSVantage SE. For cell cycle analysis, cells suspended in ice-cold ethanol overnight were pelleted by centrifugation and treated with RNase A (Sigma) for 30 min at 37°C before incubation in the dark on ice for 30 min in a solution containing PI at 50 μg/ml. The PI fluorescence of individual nuclei was measured using FACSCalibur, and cell cycle analysis was performed with ModFit LT software (Becton Dickinson).

Cell Homing Assay

Cells isolated from fetal liver were labeled for 5 min at 37°C with 3 μM Calcein-AM (Dojindo, Kumamoto, Japan). Washed cells were injected into the tail vein of 6- to 8-week-old mice. 16 (Gr-1$^+$ cells) or 4 (Ter119$^+$ cells) hr later, they were sacrificed, and cells isolated from bone marrow were stained with biotinylated anti-Gr-1 or anti-Ter119 antibody, followed by APC-conjugated streptavidin, and analyzed by flow cytometry. Based on the bright green fluorescence of Calcein labeling and APC labeling of the antibody, the numbers of transferred myeloid or erythroid cells were expressed as the amount of double positive cells.

Acknowledgments

We thank Dr. Thomas N. Sato (The University of Texas) for providing the Tie2 promoter/enhancer DNA. We also thank Drs. N. Tamai and

A. Myoui (Osaka University) for helpful comments and J. Yoshida, K. Matsumoto, and E. Yoshimura for technical assistance. This study was supported by a Research for the Future Program grant of The Japanese Society for the Promotion of Science, Sports, and Culture of Japan.

Received: December 10, 2002
Revised: May 23, 2003
Accepted: June 4, 2003
Published: August 19, 2003

References

Aiuti, A., Webb, I.J., Bleul, C., Springer, T., and Gutierrez-Ramos, J.C. (1997). The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J. Exp. Med. *185*, 111–120.

Arroyo, A.G., Yang, J.T., Rayburn, H., and Hynes, R.O. (1996). Differential requirements for α4 integrins during fetal and adult hematopoiesis. Cell *85*, 997–1008.

Arroyo, A.G., Yang, J.T., Rayburn, H., and Hynes, R.O. (1999). α4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. Immunity *11*, 555–566.

Baggiolini, M., Dewald, B., and Moser, B. (1997). Human chemokines: an update. Annu. Rev. Immunol. *15*, 675–705.

Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T.A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature *382*, 829–833.

Cumano, A., Dieterlen-Lievre, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell *86*, 907–916.

Delassus, S., and Cumano, A. (1996). Circulation of hematopoietic progenitors in the mouse embryo. Immunity *4*, 97–106.

Egawa, T., Kawabata, K., Kawamoto, H., Amada, K., Okamoto, R., Fujii, N., Kishimoto, T., Katsura, Y., and Nagasawa, T. (2001). The earliest stages of B cell development require a chemokine stromal cell-derived factor/pre-B cell growth-stimulating factor. Immunity *15*, 323–334.

Ema, H., and Nakauchi, H. (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. Blood *95*, 2284–2288.

Feng, Y., Broder, C.C., Kennedy, P.E., and Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science *272*, 872–877.

Frenette, P.S., Mayadas, T.N., Rayburn, H., Hynes, R.O., and Wagner, D.D. (1996). Susceptibility to infection and altered hematopoiesis in mice deficient in both P- and E-selectins. Cell *84*, 563–574.

Frenette, P.S., Subbarao, S., Mazo, I.B., von Andrian, U.H., and Wagner, D.D. (1998). Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow. Proc. Natl. Acad. Sci. USA *95*, 14423–14428.

Friedrich, C., Cybulsky, M.I., and Gutierrez-Ramos, J.C. (1996). Vascular cell adhesion molecule-1 expression by hematopoiesis-supporting stromal cells is not essential for lymphoid or myeloid differentiation in vivo or in vitro. Eur. J. Immunol. *26*, 2773–2780.

Harrison, D.E., Jordan, C.T., Zhong, R.K., and Astle, C.M. (1993). Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. Exp. Hematol. *21*, 206–219.

Hattori, K., Heissig, B., Tashiro, K., Honjo, T., Tateno, M., Shieh, J.H., Hackett, N.R., Quitoriano, M.S., Crystal, R.G., Rafii, S., et al. (2001). Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. Blood *97*, 3354–3360.

Hirsch, E., Iglesias, A., Potocnik, A.J., Hartmann, U., and Fassler, R. (1996). Impaired migration but not differentiation of haematopoietic stem cells in the absence of β1 integrins. Nature *380*, 171–175.

Hodohara, K., Fujii, N., Yamamoto, N., and Kaushansky, K. (2000). Stromal cell-derived factor-1 (SDF-1) acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells (CFU-MK). Blood *95*, 769–775.

Hsu, H.C., Ema, H., Osawa, M., Nakamura, Y., Suda, T., and Nakauchi, H. (2000). Hematopoietic stem cells express Tie-2 receptor in the murine fetal liver. Blood *96*, 3757–3762.

Kawabata, K., Ujikawa, M., Egawa, T., Kawamoto, H., Tachibana, K., Iizasa, H., Katsura, Y., Kishimoto, T., and Nagasawa, T. (1999). A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. Proc. Natl. Acad. Sci. USA *96*, 5663–5667.

Kim, C.H., and Broxmeyer, H.E. (1998). In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. Blood *91*, 100–110.

Lataillade, J.J., Clay, D., Dupuy, C., Rigal, S., Jasmin, C., Bourin, P., and Le Bousse-Kerdiles, M.C. (2000). Chemokine SDF-1 enhances circulating CD34+ cell proliferation in synergy with cytokines: possible role in progenitor survival. Blood *95*, 756–768.

Lataillade, J.J., Clay, D., Bourin, P., Herodin, F., Dupuy, C., Jasmin, C., and Bousse-Kerdiles, M.C. (2002). Stromal cell-derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G_0G_1 transition in CD34+ cells: evidence for an autocrine/paracrine mechanism. Blood *99*, 1117–1129.

Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T., and Springer, T.A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc. Natl. Acad. Sci. USA *95*, 9448–9453.

Ma, Q., Jones, D., and Springer, T.A. (1999). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. Immunity *10*, 463–471.

Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. Cell *86*, 897–906.

Micklem, H.S., Ford, C.E., Evans, E.P., Ogden, D.A., and Papworth, D.S. (1972). Competitive in vivo proliferation of fetal and adult haematopoietic cells in lethally irradiated mice. J. Cell. Physiol. *79*, 293–298.

Nagasawa, T. (2000). A chemokine, SDF-1/PBSF, and its receptor, CXC chemokine receptor *4*, as mediators of hematopoiesis. Int. J. Hematol. *72*, 408–411.

Nagasawa, T., Kikutani, H., and Kishimoto, T. (1994). Molecular cloning and structure of a pre-B-cell growth-stimulating factor. Proc. Natl. Acad. Sci. USA *91*, 2305–2309.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996a). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature *382*, 635–638.

Nagasawa, T., Nakajima, T., Tachibana, K., Iizasa, H., Bleul, C.C., Yoshie, O., Matsushima, K., Yoshida, N., Springer, T.A., and Kishimoto, T. (1996b). Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry coreceptor fusin. Proc. Natl. Acad. Sci. USA *93*, 14726–14729.

Nagasawa, T., Tachibana, K., and Kishimoto, T. (1998). A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. Semin. Immunol. *10*, 179–185.

Nagasawa, T., Tachibana, K., and Kawabata, K. (1999). A CXC chemokine SDF-1/PBSF: a ligand for a HIV coreceptor, CXCR4. Adv. Immunol. *71*, 211–228.

Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J.M., Clark-Lewis, I., Legler, D.F., et al. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature *382*, 833–835.

Papayannopoulou, T., Craddock, C., Nakamoto, B., Priestley, G.V., and Wolf, N.S. (1995). The VLA4/VCAM-1 adhesion pathway defines

contrasting mechanisms of lodgement of transplanted murine hemopoietic progenitors between bone marrow and spleen. Proc. Natl. Acad. Sci. USA *92*, 9647–9651.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., et al. (1999a). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science *283*, 845–848.

Peled, A., Grabovsky, V., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Petit, I., Ben-Hur, H., Lapidot, T., and Alon, R. (1999b). The chemokine SDF-1 stimulates integrin-mediated arrest of CD34+ cells on vascular endothelium under shear flow. J. Clin. Invest. *104*, 1199–1211.

Ponomaryov, T., Peled, A., Petit, I., Taichman, R.S., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Magerus, A., Caruz, A., Fujii, N., et al. (2000). Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. J. Clin. Invest. *106*, 1331–1339.

Potocnik, A.J., Brakebusch, C., and Fassler, R. (2000). Fetal and adult hematopoietic stem cells require β1 integrin function for colonizing fetal liver, spleen, and bone marrow. Immunity *12*, 653–663.

Schmits, R., Filmus, J., Gerwin, N., Senaldi, G., Kiefer, F., Kundig, T., Wakeham, A., Shahinian, A., Catzavelos, C., Rak, J., et al. (1997). CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. Blood *90*, 2217–2233.

Schlaeger, T.M., Bartunkova, S., Lawitts, J.A., Teichmann, G., Risau, W., Deutsch, U., and Sato, T.N. (1997). Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. Proc. Natl. Acad. Sci. USA *94*, 3058–3063.

Sweeney, E.A., Lortat-Jacob, H., Priestley, G.V., Nakamoto, B., and Papayannopoulou, T. (2002). Sulfated polysaccharides increase plasma levels of SDF-1 in monkeys and mice: involvement in mobilization of stem/progenitor cells. Blood *99*, 44–51.

Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., et al. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature *393*, 591–594.

Tanaka, Y., Adams, D.H., Hubscher, S., Hirano, H., Siebenlist, U., and Shaw, S. (1993). T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1β. Nature *361*, 79–82.

Vermeulen, M., Le Pesteur, F., Gagnerault, M.C., Mary, J.Y., Sainteny, F., and Lepault, F. (1998). Role of adhesion molecules in the homing and mobilization of murine hematopoietic stem and progenitor cells. Blood *92*, 894–900.

Weissman, I.L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell *100*, 157–168.

Wiesmann, A., and Spangrude, G.J. (1999). Marrow engraftment of hematopoietic stem and progenitor cells is independent of Gαi-coupled chemokine receptors. Exp. Hematol. *27*, 946–955.

Williams, D.A., Rios, M., Stephens, C., and Patel, V.P. (1991). Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. Nature *352*, 438–441.

Wright, D.E., Wagers, A.J., Gulati, A.P., Johnson, F.L., and Weissman, I.L. (2001). Physiological migration of hematopoietic stem and progenitor cells. Science *294*, 1933–1936.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature *393*, 595–599.